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Physiological Zoölogy

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THE ELECTRIC CHARGE OF PROTOPLASMIC COLLOIDS[1]

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IT IS of the very nature of colloidal systems that the individual particles or micellae of any colloidal dispersion remain separate from each other because of their electric charge. If the charge is neutralized, the particles tend to come together, and flocculation usually results. Obviously, for the understanding of the behavior of any colloidal dispersion it is a matter of prime importance to know whether the charge on the individual particles or micellae is positive or negative. In the case of the protoplasmic colloid, there is, as yet, no certain information concerning the sign of the electric charge. Ten years ago, in a summary of the information then available, one of us concluded that the charge on the micellae of the protoplasm in the interior of a typical cell was positive (Heilbrunn, 1928, chap. x). Since then, a paper of Sen has aroused wide interest and has been often quoted. Indeed, in their review of electrokinetic methods as related to biology and medicine, Abramson and Moyer (1937) copy almost all of Sen's data and comment favorably upon it. As a result of experiments on the petiole hair cells of *Urtica dioica* (stinging nettle) and on the root hairs of the aquatic plant *Azolla pinnata*, Sen concluded that the protoplasmic particles in these cells bore a negative charge.

Actually, in view of the importance of the question, the literature concerning the electric charge of the protoplasmic colloids in the interior of a living cell is very scanty. There is obviously a need for further work. In this paper we shall report observations on amoeba protoplasm, as well as observations on the protoplasm of the water plant *Elodea*. We have also reinvestigated the root hairs of *A. pinnata*, already studied by Sen.

EXPERIMENTS ON AMOEBA

When an ordinary amoeba, say an *Amoeba proteus*, is placed in an electric field so that an electric current flows through it, the amoeba moves directly and consistently toward the cathode. This is an old observation, due originally to Verworn; and it has been repeated by various other workers (for literature references see Mast, 1931, and Hahnert 1932). In order to be effective, the electric current must have a certain strength; and, on the other hand, it cannot be too strong, for with currents of too great amperage the amoeba breaks at its anodal side and dies. As the amoeba moves toward the cathode, the granules and crystals in its interior move rapidly in a cathodal direction.

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[1] This paper was supported by a grant from the Radiation Committee of the National Research Council. It is planned to follow this work with a study of the effects of radiation on the electric charge of protoplasmic colloids.

Since Verworn's original observation investigators have expressed a wide variety of opinions as to the mechanism of the galvanotropic response of the amoeba. Mast and Hahnert, in the papers cited, summarize these opinions, and they also offer suggestions of their own.

Our interest in the galvanotropic response of amoebae has been in the light it might throw on the sign of the charge on the protoplasmic colloids in the interior of the amoeba cell. The charge on granules and crystals within the amoeba is presumably conditioned largely by the charge on the protein or other colloidal micellae in which the granules or crystals are immersed. The very fact that the granules move in a cathodal direction seems to indicate that their charge can scarcely be negative. However, the granules and crystals move in a flowing mass of protoplasm, and this flow may be due entirely to some change in the boundary of the cell. Thus, if the cathodal edge of the cell were to move toward the cathode, this would pull granules and protoplasm in the same direction. Essentially, there are two possibilities. The cathodic movement of the amoeba may be due to a cataphoretic movement of granules and protoplasmic particles which push the cortex before it (and perhaps also liquefy it), or the cathodic movement of the amoeba as a whole may be due to a primary effect on the cortex or limiting membrane at the cathodal boundary of the cell.

To decide between these two possibilities is not easy. It is possible to imbed amoebae in rather stiff gelatin gels, under conditions which prevent any marked change in shape of the cell. A 10 per cent solution of gelatin is prepared, and this is then cooled rapidly. Before it stiffens, amoebae are dropped into it. The gelatin then hardens, and the amoebae are then completely surrounded by a solid gel which prevents amoeboid movement. Electric currents were passed through such imbedded amoebae, and in one or two experiments it was thought that a movement of granules toward the cathode could be observed. However, in repetitions of this experiment, it was usually impossible to obtain any satisfactory result, either because the amoeba was not completely immobilized by the gelatin or because in other instances it seems to have suffered injury.

Another type of experiment produced much more decisive results. It was reasoned that, if the charge on the protoplasmic colloids within the amoeba was positive, then it should not be difficult to neutralize or reverse this charge by the simple expedient of alkalinizing the protoplasm. As is well known from the experiments of Jacobs (1922), ammonium salts cause an entrance of ammonium hydroxide into the cell. Protoplasm can also be alkalinized by immersion of cells in solutions of ammonium hydroxide. If now, amoebae are immersed in solutions of ammonium salts or in dilute solutions of ammonium hydroxide, and an electric current is then run through the amoebae, we have a way of determining whether or not the movement toward the cathode as it occurs normally is due to a cataphoretic movement of positively charged granules and protoplasmic micellae. For, if the protoplasmic granules are normally positively charged, then addition of OH ion should decrease or even neutralize this charge, and the granules should move less rapidly or even move in the opposite direction.

Actual experiments showed that this expectation was realized. When specimens of *A. proteus* were immersed in dilute ammonium chloride solutions and then subjected to an electric current, in 48 cases out of a total number of 153, the amoebae did actually move to the anode instead of to the cathode. This is shown in Table 1. Such an anodal migration of amoebae never occurs normally, as is evidenced by the control experiments with 104 amoebae in spring water. As a matter of fact, in the numerous experiments we have done on the effect of electric current on normal amoebae, we have never seen an amoeba move toward the anode if the current was strong enough to have any effect at all on the direction of movement. Ammonium hydroxide solutions (at a pH of 8.3–9.0) also tend to cause a reversal of the normal galvanotropic response.

The simplest explanation of the galvanotropic reversal following alkalinization of the protoplasm is to assume that a normally positive charge of protoplasmic granules is changed to a negative charge. Such a reversal of charge does not occur in all specimens; and, as a matter of fact, it can barely be produced without resultant death of the amoebae. In some cells the charge is apparently neutralized, so that no directed movement occurs at all; in other cases the charge is not quite neutralized and the amoeba moves slowly toward the cathode. It should also be pointed out that, although these experiments were successful with *A. proteus*, they were not successful with the related species, *Amoeba dubia*. Apparently in the latter form it is not so easy to neutralize the charge on the granules without causing death.

TABLE 1

EFFECT OF AMMONIUM CHLORIDE SOLUTIONS ON THE DIRECTION OF MOVEMENT OF *Amoeba proteus* EXPOSED TO AN ELECTRIC CURRENT

I. CONTROL

Of 104 amoebae in culture fluid, 104 (100%) move to the cathode

II. IN NH_4Cl

Molarity of NH_4Cl	Number of Amoebae Tested	To Anode	To Cathode	Indefinite
0.0166	94	38	29	27
0.0142	35	16	12	7
0.0133	24	12	7	5
Total	153	66 (43.1%)	48 (31.3%)	39 (25.5%)

Alkalinization of the protoplasm can apparently be produced in other ways than by immersion of amoebae in ammonium hydroxide or ammonium chloride. The acidity of protoplasm is maintained by the constant production of carbon dioxide, which within the cell is in the form of carbonic acid. This carbonic acid is apparently an important factor in determining the intracellular pH. Thus, in earlier experiments, in which it was shown that alkalinization caused a release of free fat in protoplasm, it was found that an excess of carbon dioxide prevented this release, no doubt because it tended to acidify the protoplasm (Heilbrunn, 1936). Clearly then, any agents which tend to prevent carbon dioxide production (or which tend to remove carbon dioxide from the protoplasm) should tend to alkalinize the protoplasm, and they should therefore have an effect similar to that produced by the entrance of alkali within the cell. Now, it is a well-known fact that, in relatively high concentration, fat solvent anesthetics do cause a pronounced decrease in cellular respiration (for a review of the earlier literature in this field see Winterstein, 1926; references to more recent papers are given by Jowett, 1938). When amoebae are immersed in high concentrations of fat-solvent anesthetics, it is sometimes possible to demonstrate a reversal of charge in the protoplasm. Thus, when amoebae were placed in a 4 per cent ether solution and observed within a few minutes, we were occasionally able to observe a movement of protoplasmic particles and of pseudopodia toward the anode. By the time the observations were made, the concentration of ether was somewhat less than 4 per cent, owing to evaporation of the ether. Similarly, a 2 per cent ethyl urethane solution often causes such a reversal of charge, whereas 0.5 per cent and 1 per cent solutions are ineffective. This effect of high concentrations of fat solvents is interpreted as being primarily due to alkalinization.

Of course, it is possible that there are other explanations which might also account for the reversal of galvanotropism in amoeba following alkalinization. However, it should be noted that, when amoebae are immersed in ammonium chloride solutions, there is no change in the pH at the cell surface but only a change in the interior pH. Moreover, in ammonium hydroxide solutions with increase in alkalinity the surface membrane of the amoeba should become more negatively charged than ever. This would tend to increase any electroendosmotic flow toward the cathode. If, then, one assumes that galvanotropic response in amoeba is due to an electroendosmotic flow of water toward the cathode as a result of a negatively charged plasma membrane, this flow would be greater in ammonium hydroxide solutions; and on the basis of this theory one should expect a more rapid movement toward the cathode in such solutions. The simplest explanation to account for reversal of galvanotropism with alkalinization of the protoplasm is to assume that the original positive charge of the protoplasmic particles is changed to a negative charge. This was the prediction made before the experiment was tried, and the results serve to bear out the prediction. As the granules move either toward cathode or anode, they must tend to break down the thixotropic gel on the side toward which they move. That the cortex of the amoeba is a thixotropic gel is shown by the experiments of Angerer (1936). If this gel is liquefied in any local region, such a region becomes pushed out to form an advancing pseudopod. At any rate, this is the usual interpretation given by modern theories of amoeboid movement.

It seems clear that our experiments on amoeba lend support to the view that the protoplasm in the interior of the amoeba is a positively charged colloid.

EXPERIMENTS ON *Elodea*

In experiments with amoeba the changes in shape of the cell introduce difficulties. Plant cells typically are inclosed by a rigid cell wall, so that the boundaries of the cell remain in a constant position. However, in dealing with plant material it is not always a simple matter to determine when the protoplasm is injured or dead. In many plant cells the protoplasm constantly streams; and this streaming, or cyclosis, ceases on violent injury or death. Accordingly, such cells with streaming protoplasm offer advantages for the solution of our problem, for it is possible to adjust the strength of the electric current so that it will cause an effect and yet not produce death.

The well-known water plant, *Elodea canadensis*, has been widely used in studies of protoplasm and is readily available. The protoplasm in the leaf cells of this plant may stream when the leaves are on the plant. This streaming tends to become more rapid, and it is of more regular occurrence after the leaves have been cut away. In our experiments leaves were cut from the stem and allowed to remain in water for at least 1 hour before they were studied. The current was led to the leaves through agar bridges from nonpolarizable zinc–zinc sulphate electrodes. The electrodes were connected with a 110-volt circuit, and the amperage was then varied by inserting suitable resistance.

In currents not quite strong enough to cause stoppage of protoplasmic streaming the rate of movement of the chloroplasts is affected by the electric current. This was determined by noting the speed of the chloroplasts as they moved around the cell. In the one direction they traveled toward the cathode; and in the other, toward the anode. We would have preferred to measure the speed of the tiny protoplasmic granules rather than the speed of the chloroplasts, but such measurement is very tedious and technically well-nigh impossible for a long series of experiments. As a matter of fact, the measurement of the speed of chloroplasts is not too easy, for one must make a series of measurements for the same cell before and after the application of the electric current. It is essential to select a cell for measurement which does not have too many chloroplasts, for when these structures are very numerous, they tend to get in each other's way. The result is that the movement may become jerky and irregular, and measurements under these conditions are not very satisfactory.

In measuring the movement of chloroplasts rather than protoplasmic granules, we were at a disadvantage. Although the electric charge of the chloroplast is in large measure determined by the charge of the micellae of the protoplasm in which it is bathed, one cannot deny that the essential nature of the chloroplast itself may be a factor. Our results show the sign of the electric charge at the surface of the chloroplast, and it is something of an assumption to suppose that this charge is the same as that of the rest of the protoplasm. At the worst, we do obtain information concerning the chloroplast charge.

The experiments were performed in the following manner. A detached *Elodea* leaf, or a section of such a leaf, was placed in spring water between two agar bridges leading to the electrodes. The leaf was oriented so that the current flow was in a direction parallel to the midrib; in other words, the current flowed through the length of the cells of the leaf rather than across them. With the leaf in position between the agar bridges, a favorable cell was chosen. When this cell was viewed in optical section, the chloroplasts could be observed moving in a given direction along one of the long walls and in an opposite direction along the opposite wall. Their speed along each of these walls was then determined with the aid of an ocular micrometer eyepiece and a stop watch. Usually, the scale of the ocular micrometer could be moved first to a position over one wall of the cell and then over the opposite wall by the simple expedient of rotating the ocular of the microscope. Typically, ten measurements were made on each side, and these were averaged. In general in these control experiments the average speed of chloroplast movement on the two sides of the cell was approximately the same, and only rarely did the difference in rate of speed exceed a value of 15 per cent. Once we were sure that the cell was behaving with reasonable normality and that the speed of chloroplast movement on opposite walls was not widely different, the electric current was applied. During the time that the electric current was flowing, the chloroplasts were moving with the current on one side of the cell and against the current on the opposite side. With the electric current flowing, measurements of the rate of movement of the chloroplasts on the two sides of the cell were made with the same technique as that used before the electric current was turned on. In many cases it was obvious that the electric current was having an effect, for the difference in rate of chloroplast movement on the two sides of the cell was often much greater than 15 per cent. Not infrequently, differences as great as 30 or 40 per cent were obtained. The results of these experiments are given in Table 2. They were very disappointing. It had been hoped that the speed of the chloroplasts would be greater either to the cathode or to the anode. Actually, in some experiments the speed was greater toward the cathode; in some other cases it was greater toward the anode. Inspection of Table 2 shows sixteen cases of greater speed toward the cathode and twenty-two cases of greater speed toward the anode. To these twenty-two cases

TABLE 2

EFFECT OF AN ELECTRIC CURRENT ON THE SPEED OF CHLOROPLASTS IN *Elodea* CELLS

No. of Expt.	Rate of Streaming in Control			Rate of Streaming during Passage of Current			Strength of Electric Current (in Milliamperes)
	To Right (μ/Sec.)	To Left (μ/Sec.)	Percentage Difference	Toward Anode (μ/Sec.)	Toward Cathode (μ/Sec.)	Percentage Difference	
1	6.5	6.1	6.5	6.1	4.8	27.0	5.9
2	3.7	3.6	2.7	3.5	4.9	40.0	2.0
3	7.6	7.6	....	10.4	6.9	50.7	3.0
4	5.2	5.7	9.6	5.7	4.1	39.0	2.0
5	5.9	5.5	7.2	4.6	6.3	36.9	3.0
6	5.5	6.0	9.0	6.9	4.9	40.8	6.0
7	5.2	4.5	15.5	3.8	5.0	31.5	4.0
8	5.3	5.8	9.4	6.5	5.6	16.0	4.0
9	4.1	4.3	4.8	4.6	3.3	39.3	5.0
10	2.9	2.6	1.1	2.9	3.8	31.0	2.0
11	5.3	4.6	15.2	3.8	3.7	2.7	2.0
12	5.9	6.0	1.6	6.3	4.3	46.5	3.0
13	4.0	4.0	....	2.7	3.3	22.2	2.0
14	5.7	6.1	7.0	4.4	4.1	7.3	2.0
15	3.8	4.0	5.2	4.2	3.4	23.5	2.0
16	5.6	6.3	12.5	6.5	4.1	58.5	2.0
17	5.6	5.9	5.3	6.1	4.5	35.5	1.0
18	6.6	5.8	13.7	6.1	6.9	13.1	1.0
19	5.8	6.5	12.0	7.4	5.8	27.5	2.0
20	5.1	4.9	4.0	6.3	4.9	28.5	1.0
21	3.9	4.0	2.5	4.5	3.7	21.6	0.5
22	2.5	2.5	....	3.2	2.2	45.4	2.0
23	4.3	3.9	10.2	3.6	5.0	38.8	1.25
24	5.8	5.4	7.4	5.6	4.0	40.0	6.7
25	6.1	5.9	3.3	3.9	2.4	62.5	6.9
26	4.0	4.1	2.5	3.2	4.6	43.7	5.4
27	3.2	3.3	3.1	2.9	2.4	20.8	2.5
28	2.8	2.9	3.5	2.5	2.8	12.0	2.0
29	6.0	6.5	8.3	6.4	8.2	28.1	4.0
30	6.0	7.4	23.3	6.0	3.4	76.4	3.0
31	4.6	4.7	2.1	3.9	3.1	25.7	1.0
32	7.8	7.7	1.2	8.2	7.0	17.0	0.1
33	7.3	7.0	4.2	5.8	8.0	37.9	0.15
34	6.7	7.1	5.9	5.9	9.2	55.9	0.15
35	4.6	4.2	9.5	5.4	4.7	14.8	0.1
36	5.4	5.6	3.7	3.2	4.6	43.7	0.125
37	6.4	6.8	6.2	5.9	7.1	20.3	0.125
38	5.7	5.5	3.6	4.9	6.4	30.6	....
39	5.5	5.4	1.8	6.5	5.9	10.1	0.15
40	6.4	5.6	14.4	4.9	5.8	18.3	0.175

might be added two other cases in which there was a greater speed toward the anode but in which the difference in speed seemed insignificant (less than 10 per cent). The whole experiment might have seemed futile if it were not for the fact that in many cases the electric current appeared to be exerting a very real influence. Thus, in one instance the speed of chloroplasts was 76 per cent greater when the chloroplasts were traveling toward the anode than was the speed when the chloroplasts were traveling toward the cathode. In another case there was a 56 per cent difference in favor of the movement toward the cathode.

Therefore, the results apparently indicate that the chloroplasts are actually charged; but if we are to draw any conclusions, it would seem to be true that they are charged sometimes positively and sometimes negatively. At first we were at a loss to account for this apparent difference in charge in different experiments, and we made a number of efforts to conduct our experiments in such a way as to show that the chloroplasts are always charged either positively or negatively. Thus the experimental technique was varied by cutting the leaves in different ways; but no matter which type of leaf preparation was used, there was no constancy in the charge of the chloroplasts as determined by the relative speed of their movement toward cathode or anode.

Finally, we were led to believe that the chloroplasts might be charged either positively or negatively and that the sign of the charge depended on the intensity of photosynthesis in the cell. This point of view seemed logically plausible, for during photosynthesis carbonic acid is being rapidly removed from the protoplasm, whereas in the absence of photosynthesis the protoplasm can be assumed to be drenched with carbonic acid. The presence of more or less carbon dioxide may have a profound influence on the hydrogen ion concentration of the protoplasm. This point has already been discussed in relation to the experiments with amoeba. Accordingly, we might expect that during active photosynthesis the protoplasm in general, and the surface of the chloroplast in particular, would tend to be more negatively charged, owing to the absence of acid carbon dioxide. On the other hand, when photosynthesis is not occurring, the presence of carbon dioxide would tend to make the charge on protoplasmic micellae and chloroplasts positive.

Thus, if one could eliminate photosynthesis, the charge on the particles within the protoplasm might always be positive. For a time we tried to make measurements of chloroplast speed in almost complete absence of light. This did not prove practicable. Indeed, it is difficult enough to obtain measurements even with the best illumination possible. We looked then for ways of inhibiting photosynthesis in the light. Years ago, Claude Bernard showed that dilute solutions of fat-solvent anesthetics could prevent photosynthetic activity in green plants without having much effect on respiration, and this work has had the support of later experimenters (for literature references see Winterstein, 1926, pp. 138–41). In general, it may be said that there is a rather narrow range of concentration in which fat solvents, such as chloroform, depress photosynthesis without depressing respiration. Using the technique already described, we studied the influence of electric current on chloroplast speed in leaves immersed in dilute solutions of chloroform and of ethyl urethane. We hoped that exposure to these anesthetics would give us uniform results and that in these experiments the chloroplasts would always behave as if positively charged. Again we were disappointed, for, as before, the chloroplasts were sometimes charged positively and sometimes negatively. It is quite possible that we did not select the exact concentration of anesthetic necessary to inhibit photosynthesis without changing the rate of respiration, and this may account for our failure to obtain consistent results.

Clearly we needed more information as to the conditions under which photosynthesis might be inhibited in our *Elodea* material in the light. Modern studies of photosynthesis use standard analytical methods for the determination of oxygen liberated or carbon dioxide utilized. In general, however, these newer methods confirm the older observations made simply by counting the number of bubbles of oxygen given off by aquatic plants such as *Elodea*. We were in search of only qualitative information, and for this reason we adopted the simple technique of Sachs for watching the course of photosynthesis. An ordinary funnel was inverted under water, and its tube was suitably covered with a test tube also filled with water. *Elodea* plants were then imprisoned under the funnel. The oxygen bubbles released as a result of photosynthetic activity passed up into the test tube, displacing the water at the upper end of the test tube. From time to time the volume of oxygen gas in the test tube could be measured. With such a simple arrangement we were able to note two sets of conditions in which photosynthesis was inhibited. In the first place, although photosynthesis was very active for the first day during which the *Elodea* plants were imprisoned under the funnel, the rate of photosynthetic activity, as measured by gas production, fell off rapidly, so that after several days there was no increase in the volume of oxygen in the test tube; and at this time, bubbles were no longer given off by the plants. Secondly, after *Elodea* leaves had been kept in the dark for several days, their photosynthetic activity did not return immediately; or, at any rate, in the case of such plants removed from the dark the evolution of oxygen was, for a time, very slow.

Our first experiments were on the leaves of plants which had been imprisoned under funnels for several days. Why photosynthesis should have stopped in these leaves we do not know. The fact remains, however, that it did stop. Measurements of chloroplast speed were then made on these leaves which, while under the funnel, had ceased to show photosynthetic activity. A total of twenty-three series of measurements were made. In every case the chloroplasts showed more rapid movement toward the cathode, indicating that in every case they were charged positively. Although these experiments gave us the result we had hoped to obtain, we were not very content with them, largely because we could not understand why imprisonment under the funnels should cause cessation of photosynthesis. We therefore turned to the second type of condition in which photosynthesis is retarded even though the plant cells are in the light.

Elodea plants were kept in the dark for 6–9 days; the leaves were then cut off and were studied with our usual technique. Photosynthetic activity tends to begin soon after the leaves emerge into the light. Accordingly, it was necessary to make measurements of chloroplast speed as rapidly as possible. Usually the measurements were completed within about a half-hour after exposure to light was begun. Moreover, in making the measurements, light passing through the leaves was cut down to some extent. Before the light entered the leaf it usually passed through a green filter, and this served to cut down the intensity of the radiation. The results obtained in experiments with leaves previously kept in the dark are shown in Table 3. They are wholly consistent. In all, the chloroplast speed was measured in twenty-six leaf preparations. In twenty-four of these preparations the chloroplasts moved toward the cathode with a speed significantly greater than that they showed toward the anode. In the other two preparations, one showed no difference in rate of speed toward cathode or anode while the other showed an increase in rate of speed toward the cathode; but this was not significant, for the difference was not appreciably greater than that shown in the control (before the current was applied). In no single experiment was the chloroplast speed greater toward the anode, so that in 100 per cent of those leaves which gave a clear answer, the speed of the chloroplasts was greater toward the cathode. Obviously, under the conditions of the experiment, the chloroplasts bore a positive charge. It should be emphasized once more that these measurements must be made reasonably soon after the leaves emerge into the light. In a number of cases the cells were studied again after they had remained in the light for a half-hour or longer. Under these conditions the chloroplasts sometimes showed a positive, sometimes a negative, charge.

TABLE 3

EFFECT OF AN ELECTRIC CURRENT ON THE SPEED OF THE CHLOROPLASTS IN *Elodea* LEAVES WHICH HAVE BEEN KEPT IN THE DARK

No. of Expt.	Rate of Streaming in Control			Rate of Streaming during Passage of Current			Strength of Electric Current (in Milliamperes)
	To Right (μ/Sec.)	To Left (μ/Sec.)	Percentage Difference	Toward Anode (μ/Sec.)	Toward Cathode (μ/Sec.)	Percentage Difference	
1	4.5	4.7	4.4	2.9	3.6	24.1	0.175
2	5.4	5.1	5.8	4.3	5.5	27.9	0.175
3	5.0	5.0	....	4.8	5.9	22.9	0.175
4	4.2	4.3	2.3	3.9	4.9	25.6	0.175
5	5.1	5.1	....	4.3	6.1	41.8	0.175
6	9.5	8.9	6.7	7.6	10.1	32.8	0.2
7	8.0	7.3	9.5	3.4	5.8	70.5	0.325
8	4.9	5.2	6.1	4.5	5.6	24.4	0.9
9	6.3	6.3	....	5.5	7.6	38.1	0.875
10	5.2	4.9	6.1	4.7	6.3	34.0	0.95
11	3.8	4.0	5.2	3.7	5.1	37.8	0.2
12	4.8	4.4	9.0	4.0	5.9	47.5	0.175
13	3.1	3.2	3.2	2.9	4.0	37.9	0.2
14	3.8	3.8	....	3.9	4.3	10.2	0.15
15	4.7	4.8	2.1	3.6	4.1	13.8	0.15
16	5.9	5.5	7.2	5.2	5.6	7.6	0.15
17	3.1	3.1	....	2.8	3.3	17.8	0.2
18	4.8	4.7	2.1	3.8	4.3	13.1	0.2
19	2.9	2.8	3.5	2.8	3.6	28.5	0.2
20	4.3	4.6	6.9	3.9	5.2	33.3	0.2
21	3.1	3.0	3.3	2.4	3.7	54.1	0.2
22	2.8	3.2	14.1	2.8	2.8	....	0.2
23	3.0	3.5	16.6	2.5	3.1	24.0	0.175
24	3.0	3.4	13.3	2.3	3.5	52.1	0.2
25	3.3	3.2	3.1	2.7	3.4	25.9	0.175
26	4.1	3.8	7.8	4.0	5.7	42.5	0.175

It may, with reasonable safety, be concluded that in the dark, or under conditions which tend to inhibit photosynthesis, the electric charge on the chloroplasts, and presumably also on the protoplasmic micellae of the *Elodea* cell, is positive. There is thus additional evidence that the normal charge of the protoplasmic colloid is positive. It is a matter of considerable interest that this charge may be reversed in sign by photosynthesis.

EXPERIMENTS ON *Azolla*

The results thus far obtained have been wholly consistent with the thesis that the colloidal particles of protoplasm bear a positive charge. How, then, are we to interpret the results of Sen, already mentioned? It will be remembered that Sen investigated the petiole hair cells of *U. dioica* and the root-hair cells of *A. pinnata*. It is the results with *Azolla* cells that are especially interesting, for in these cells Sen was able to give quantitative figures for the cataphoretic movement of particles within the cells. Abramson and Moyer thought these figures so important that they recopied them in their 1937 paper.

In his discussion Sen states that "the particles very near the cell walls scarcely moved, and, if at all, toward the cathode." Particles farther away from the cell walls moved toward the anode, and those about midway between the cell walls showed the maximum velocity. In thinking over these observations it occurred to us that the particles which Sen observed moving toward the anode might be in the cell vacuole rather than in the cell protoplasm. Indeed, a statement to this effect was made in a recent book (Heilbrunn, 1937, p. 77). However, Sen states clearly that he was studying protoplasmic particles. It was thought worth while, therefore, to examine the root-hair cells of *Azolla* in order to determine whether or not the particles studied by Sen were really protoplasmic or not.

The root hair of *Azolla*, viewed under dark-field illumination, is a beautiful object. The granules in the center of the cell are in active Brownian movement. As soon as they are subjected to an electric current, they show a rapid cataphoresis. When the direction of the current is reversed, the granules also reverse their movement. In every case they move toward the anode. It is thus a simple matter to confirm Sen's observations. However, there is a question as to whether the granules which show such beautiful Brownian movement and which move so readily toward the anode are really in the protoplasm. Sen states that the *Azolla* root hair is "practically a cellulose capillary of uniform diameter filled with transparent protoplasm." But if one examines Sen's figures, especially his Figure 1, the cell appears like a typical root-hair cell with a narrow layer of protoplasm surrounding a central vacuole which occupies the major portion of the cell. Is this the true structure? In order to settle this point, root hairs were stained with neutral red. In such stained cells the red color was largely confined to a narrow layer beneath the cell wall. However, this could hardly be regarded as conclusive proof that the center of the cell is a vacuole. Much better evidence is given by experiments in which the cells were treated with 70 or 95 per cent alcohol. After *Azolla* preparations had been thoroughly fixed in such alcoholic solutions for 4–12 hours, the granules in the center of the cell retained their Brownian movement and moved toward the anode, just as they did in the living preparations. Obviously, following fixation with alcohol, Brownian movement in protoplasm ceases, and cataphoretic movement of protoplasmic particles would also be inhibited.

There is this difference between the behavior of the *Azolla* cells fixed in alcohol and living *Azolla* cells. When an electric current is run through the living cells, the protoplasmic layer beneath the cell wall seems to grow thicker, and it apparently becomes sticky. The result is that the actively moving particles in the center of the cell after a time cease Brownian movement and no longer move under the influence of the electric current. However, when an electric current is run through cells fixed in alcohol, the granules in the center of the cell continue to respond to the electric current for hours. Sometimes after a lengthy exposure the granules become aggregated into balls whose diameter is perhaps two-thirds of the entire cell. Under the influence of the electric current these balls can be seen to move back and forth as the direction of the electric current is changed. Like the granules of which they are composed, the balls move rapidly toward the anode. Anyone observing these balls rolling through the central region of the *Azolla* root hair could scarcely doubt but that this is a typical root hair and possesses a well-developed vacuole.

It is thus clear than Sen's observations on the granules in the center of *Azolla* cells were made on particles within the cell vacuole. They thus offer no information concerning the charge on protoplasmic particles. It will be remembered that for particles near the cell wall, thus presumably in the protoplasm, Sen observed a movement toward the cathode.

DISCUSSION

Our results with the amoeba and with *Elodea* bear out the opinion expressed earlier (Heilbrunn, 1928) that the charge of the particles of the protoplasmic colloid is typically positive. This charge is apparently dependent on the concentration of carbon dioxide present in the protoplasm. If this carbon dioxide is reduced in amount, as it may be in photosynthesis, the sign of the charge may be reversed.

In addition to the data which we have presented, there is another observation which indicates a positive charge of protoplasmic colloids. Churney and Klein (1937) passed electric currents through the large salivary gland cells of the larva of the fly *Sciara*. As a result of the passage of the electric current the chromatin within the nucleus moved toward the anode, but the nucleus as a whole moved toward the cathode. Although it was unknown to Churney and Klein at the time of their experiments, a similar observation was made by Dahlgren in 1915. He passed electric currents through nerve cells of the electric ray (*Torpedo ocellata*) and observed a movement of the nuclei toward the cathode. Dahlgren's observations are illustrated in his Figures 19 and 20. He was not interested in determining the charge on protoplasmic colloids, and he makes no mention of these experiments in the summary of his paper. It seems possible that the cells of electric fishes would constitute favorable material for the study of cataphoretic effects, for the protoplasm of these cells is no doubt accustomed to the passage of an electric current and would not be so readily killed as is the protoplasm of a typical cell.

A cell nucleus might well be assumed to have its surface impregnated with the colloids of the cytoplasm. Hence, presumably its surface would bear the same charge as that of the protoplasmic micellae. It is consistent with our thesis, therefore, that Churney and Klein, and also Dahlgren, should observe a movement of the nucleus as a whole toward the cathode.

In undertaking our experiments with the amoeba, our primary purpose was to investigate the charge on the micellae of the protoplasm. Viewed from another standpoint, however, our results can be taken to serve as an explanation of the galvanotropic behavior of the amoeba. Indeed, they constitute a rather complete mechanical explanation of this galvanotropism. Moreover, it is possible to interpret reversal of the tropistic response as caused by fat solvents.

SUMMARY

1. The movement of an amoeba toward the cathode is due to the positive charge of its protoplasmic particles. This is indicated by the fact that, when the protoplasm is

made alkaline by treatment with NH_4OH or NH_4Cl, the particles within the amoeba and the amoeba as a whole may often be caused to migrate toward the anode.

2. The charge on protoplasmic micellae is thus dependent on the pH of the cell interior.

3. When amoebae are placed in concentrated solutions of fat solvents, they tend to move toward the anode, indicating that the charge on their protoplasmic colloids has been reversed. This is believed to be due to an effect of these concentrated solutions of fat solvents on the rate of respiration. With decrease in carbon dioxide in the protoplasm the colloidal particles in the protoplasm tend to assume a negative charge.

4. When an electric current is sent through the streaming protoplasm of an *Elodea* cell, the chloroplasts sometimes tend to move more rapidly toward the cathode; sometimes they move more rapidly toward the anode. The results are taken to mean that the charge on the chloroplasts may be either positive or negative.

5. Under conditions which tend to inhibit or retard photosynthesis, the chloroplasts of *Elodea* were found always to move more rapidly toward the cathode.

6. The conclusion to be drawn is that the typical charge of the micellae of the protoplasmic colloid is positive. This positive charge may be reversed by suitable alkalinization of the protoplasm. Such alkalinization may be produced (*a*) by the direct addition of alkali to the protoplasm; (*b*) by reduction in the amount of carbon dioxide in the protoplasm as a sequence of a lowered rate of respiration; (*c*) by reduction in the amount of carbon dioxide in the cell as a result of photosynthesis.

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THE VOLUMES OCCUPIED BY THE FORMED CYTOPLASMIC COMPONENTS IN MARINE EGGS[1]

(One plate)

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THE visible formed components in living cells, including the oil drops, yolk spheres, pigment granules, mitochondria, Golgi bodies, and the like, constitute, together with the hyaline ground substance, a heterogeneous coarse suspension. While it is not possible to characterize any of these elements as "living," in contradistinction to "lifeless," constituents of the protoplasm, nevertheless there is reason to conclude that, of all the cell constituents, the clear hyaloplasm is the most constant and the most active, and may perhaps be regarded as forming the fundamental basis of the protoplasmic system (Wilson, 1925, p. 78).

Information concerning the relative quantities of the formed components in different types of cells must be considered in the investigation of numerous problems. The direct chemical analysis of protoplasm may be first mentioned. Chemical analyses of protoplasm are not directly comparable, since investigators have made use of cells of varying types, containing unknown proportions of these formed components, some of which may perhaps be considered as inert cell products rather than as active constituents of living substance. Indeed, if the chemical composition of protoplasm in many different cells or organisms should happen to be the same, the varying proportions of inert products also included in the analyses would obscure this identity.

Information regarding the relative volumes of visible formed components has been found useful in the solution of other problems. Heilbrunn (1926) made use of the calculated relative volumes of yolk and pigment granules in his determination of the viscosity of the *Arbacia* egg. E. N. Harvey (1932) calculated the relative volumes of the several layers of substances in the centrifuged *Arbacia* egg. These calculations, together with unpublished data of the author for the egg of *Echinarachnius*, were used by Holter (1936) in ascertaining the place of action of the peptidases in these eggs. Holter also calculated the relative volumes of the dispersed substances in the egg of *Chaetopterus*.

The widespread occurrence of intracellular inclusions, such as granules and vacuoles, which frequently take on a color with dyes or which possess natural pigments, makes it necessary to consider them in relation to the pH or rH of protoplasm. Chambers

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[1] This study was begun in 1932. A part of the work was performed during the tenure of a National Research Fellowship in the Biological Sciences (1934–35). Measurements on the eggs of *Anisodoris, Archidoris, Arenicola, Cadlina, Diaulula, Diplodonta, Hopkinsia, Mytilus, Polychoerus, Rostanga, Strongylocentrotus, Triopha,* and *Urechis* were made at the Hopkins Marine Station of Stanford University. All other measurements were made at the Marine Biological Laboratory, Woods Hole, Massachusetts. I am indebted to Dr. W. K. Fisher, director of the Hopkins Marine Station, and to Dr. M. H. Jacobs, director of the Marine Biological Laboratory, for providing facilities for the work; also to Dr. C. V. Taylor for reading the manuscript.

(1929) has already discussed this problem in detail. The separation of the different types of granules by the use of the centrifuge, however, reveals with the greatest delicacy the location of injected or absorbed dyes.

Furthermore, there is an indirect relationship between the relative volumes of formed substances and the osmotic properties of cells. The fate of the water that enters a living cell from a hypotonic solution is not well understood. Some authors maintain that it occupies, for a period of time, discrete vacuoles within the cytoplasm. Few cells behave as perfect osmometers, a fact generally attributed to the presence within most cells of osmotically inactive substances. McCutcheon, Lucké, and Hartline (1931) investigated the osmotic properties of unfertilized *Arbacia punctulata* eggs in sea water of various concentrations. Employing a formula of Hamburger (1898) for estimating the osmotically inert material, they found that, if one takes into consideration a quantity of dispersed substances occupying 7–14 per cent of the egg volume, the product of the volume of the eggs and the osmotic pressure of the external solution is almost constant. In addition, Białaszewicz (1933), likewise applying the principle of Hamburger to the study of unfertilized eggs, found that the volume of the dispersed phase ("espace mort," "Protoplasmagerüst") were as follows for the eggs of four echinoderms: *Phallusia mamillata*, 19.3 per cent; *Echinus microtuberculatus*, 22.6 per cent; *Paracentrotus lividus*, 25.1–27.0 per cent; *Arbacia pustulosa*, 36.4 per cent. Białaszewicz' results were consistent only for hypertonic solutions, because in hypotonic solutions there was "un gonflement des colloïdes oöplasmiques qui résulte de la dilution des électrolytes dans le liquide intermicellaire." The osmotic properties of the egg may well be influenced by the relative quantities of the formed components. That is, the volume of the dispersed phase may correspond in part to the volume occupied by the cytoplasmic inclusions. Measurements of the volumes occupied by the formed components in the normal egg are preliminary to experiments upon the volumes occupied by these same components in solutions of various osmotic pressures.

Finally, the investigation of the nature of living substance, to ascertain whether it is colloidal or noncolloidal, should make use of the hyaline ground substance only, free of all extraneous material, such as visible granules and vacuoles (Just, 1936). A study of the relative amounts of such formed components in many types of cells should facilitate the selection of material suitable for such an investigation.

METHODS

The separation of the several types of substances in the living egg cells was brought about by means of the centrifuge. Any egg cell subjected to a sufficiently great centrifugal force for an adequately long period showed a stratification of the cell contents. After removal from the centrifuge tubes, the eggs were photographed at right angles to the axis of centrifuging. With the aid of the photographs the volumes of the different strata were calculated. Two methods of calculation were employed. First, the volumes of the spherical segments of known height were obtained by direct geometrical computation. Second, a graph was constructed, plotting height of the spherical segment, as a function of the egg diameter, against segment volume. This resulted in a sigmoid curve, from which volumes of spherical segments of known height could be read off, thus obviating the necessity of individual computations.

The errors involved in the determination of the volumes occupied by the formed components of the egg are many and difficult of evaluation. Errors may arise as a result of (1) insufficient packing of the granules, owing to (*a*) employing too low a centrifugal force, (*b*) centrifuging for too short a time, or (*c*) slight redistribution of granules before photographing; (2) differences in the diameter of the granules of a given segment, making difficult the estimation of the free space (i.e., hyaloplasm) between the packed granules (26 per cent for incompressible granules of uniform diameter); (3) nonsphericity of the centrifuged egg, owing to the elongation produced by the separation of centripetally directed oil from centrifugally directed yolk, rendering inaccurate the calculation of the volumes of the segments. To these errors should perhaps be added the unknown factors introduced by possible electrokinetic charges upon the cytoplasmic granules and by distortion of the shape of the granules under high centrifugal forces.

Some of the foregoing listed sources of error may be eliminated. It is possible to centrifuge eggs at various magnitudes of centrifugal force for varying periods of time. Providing that the egg does not cytolyze or fragment under these conditions, the limit of granule-packing may thereby be determined. It is also possible to observe the rate of redistribution of the granules in the centrifuged eggs and to allow for the amount of redistribution that has occurred at the time the photograph is taken. Unfortunately, it is not possible to estimate the amount of free space between the packed granules other than by comparison of the volumes obtained by varying centrifugal treatments. There is no doubt but that the granules in any one stratum vary greatly in size. Since it is therefore impossible to apply a correction designed for granules of uniform diameter, the measurements are recorded in this paper in terms of the volumes occupied by the several strata of material, without allowing for the quantity of hyaline protoplasm filling the interstices between the granules of these various layers. Nonsphericity of the entire egg may be estimated and allowed for in the calculations of the volumes of the spherical segments.

Since the measurements recorded in the present paper have been accumulated over a period of years, using many different types of centrifuges, it has not always been possible to subject the experimental material to a sufficient range of centrifugal forces to fulfil all these conditions. The maximum centrifugal force employed for each egg species will therefore be included in the data. It is quite probable that the application of increasingly high centrifugal forces to these same species of eggs would yield results of a higher order of accuracy. Some eggs, however, would cytolyze under the influence of higher centrifugal forces.

RESULTS

The results are presented in Table 1 in terms of the relative volumes of the centrifuged egg occupied by the spherical segments of the different substances. In column 1 are listed the species of eggs studied; in column 2, the volume, in percentage of total egg volume, of the centripetally located fat or oil zone; in column 3, the volume of the hyaline segment; in column 4, the volume occupied by the "yolk" granules; in column 5, the volume occupied by other types of granules; in column 6, the volume of the germinal vesicle, if present; and in column 7, the maximum centrifugal force employed.

The oil or fat zone is the most centripetally located segment in each centrifuged egg. When only one hyaline zone is present, this segment is just centrifugal to the oil layer, and contains, at the appropriate stage of egg maturation, the germinal vesicle or the nucleus. Frequently the maturation spindle is also thrown into this segment. When two zones of hyaloplasm are present, one is at the position just mentioned and the second is between the yolk segment and the segment occupied by the heaviest granules of the egg. This condition was found in *Echinarachnius*, *Polychoerus*, and strongly centrifuged *Nereis* eggs. There is a possibility that this second hyaline zone may have resulted from the breakdown (cytolysis) of some of the heavier yolk spheres, although this is unlikely. Where only three zones of stratification are present, the zone of the yolk spheres is centrifugal to hyaline zone I. That is, it is the most centrifugally located segment of the egg. In the yolk segment of certain eggs superficially showing only three layers (oil, hyaline zone, and yolk), there may be distinguished granules of different size or density that have not been completely segregated by centrifuging. The heaviest yolk

TABLE 1

RELATIVE VOLUMES, IN PERCENTAGES, OCCUPIED BY THE FORMED COMPONENTS OF EGGS CENTRIFUGED AT INDICATED FORCES*

Species	Relative Volumes (per Cent)					Maximum Centrifugal Force (Times Gravity)
	Oil	Hyaline Zone	Yolk	Other Granules	Germinal Vesicle	
Amphitrite ornata	25	38	31		6	6,000
Anisodoris nobilis (fertilized)	3	29	68			270,000
Arbacia punctulata	2	45	38	10+5		6,000
Archidoris montereyensis (fertilized)	3	30	67			240,000
Arenicola claparedii (fertilized)	15	35	50			4,800
Asterias forbesi	3	20	77			6,000
Cadlina flavomaculata (fertilized)	3	30	67			265,000
Cerebratulus lacteus	5	52	35		8	6,000
Chaetopterus pergamentaceus	7	48	45			6,000
Cumingia tellinoides	10	42	35	13		20,000
Diaulula sandiegensis (fertilized)	3	31	66			265,000
Diplodonta orbella (fertilized)	25	55	20			150,000
Echinarachnius parma	1	I—10 II—10	60	19		6,000
Glycera sp.	8	42	50			6,000
Hopkinsia rosacea (fertilized)	2	31	67			100,000
Hydroides hexagonus	10	60	30			6,000
Mytilus californianus	14	55	31			4,800
Nereis limbata (unfertilized)	9	33	55		3	6,000
N. limbata (fertilized)	8	I—16 II— 9	55	9+3		240,000
N. limbata (fertilized)	9	36	55			6,000
Phascolosoma gouldi	13	40	47			6,000
Polychoerus carmelensis (fertilized)	8	I—15 II— 3	60	4+10		100,000
Rostanga pulchra (fertilized)	5	20	75			100,000
Sabellaria vulgaris	14	53	33			6,000
Strongylocentrotus purpuratus	2	28	70			50,000
Styela partita	4	26	60	6	4	6,000
Triopha carpenteri (fertilized)	3	38	59			100,000

* These volumes are not corrected for the hyaloplasm occupying the interstices between the granules. The eggs are unfertilized unless otherwise indicated.

granules are usually identical in staining reaction with the erythrophilous (neutral-red staining) granules, and in many cases only by centrifuging stained eggs can the stratum of ordinary yolk granules be distinguished from the stratum of the heavier erythrophilous yolk granules. In the egg of *Asterias forbesi*, for example, staining with neutral red reveals erythrophilous granules at the centrifugal pole of the yolk zone, which are indistinguishable from yolk granules in unstained eggs. In the egg of *Rostanga pulchra* the yolk granules are of two types, colorless and orange, and the orange granules form a fairly distinct segment centripetal to the colorless ones. These pigmented yolk granules give the characteristic color to the egg ribbon. In certain lots of *Phascolosoma* eggs, yolk granules of dark brown and of pale brown were to be distinguished, forming indistinct zones. Where these different types of yolk granules were incompletely separated from each other with the centrifugal technique employed, they were included in Table 1 as making up the single "yolk zone." Under the heading "Other Granules" are included those granules that were distinctly separated from the yolk spheres or were of an obviously different type. The fifth layer of the *Arbacia* egg (constituting 5 per cent of the egg volume) is centripetal to the colorless yolk layer at the border of the hyaline zone (E. B. Harvey, 1932), and the erythrophilous granules are the heavy red-pigment granules (constituting about 10 per cent of the egg volume) containing echinochrome. In *Urechis* the cerise heme granules are the heaviest substance of the egg (Taylor, 1931). In the egg of *Cumingia*, the pink pigment granules are located centrifugally to the colorless yolk granules of smaller size (Costello, 1934). In these cases the additional zones of granules are included in Table 1 as "Other Granules."

In the egg of *Styela*, as shown in Plate 1, Figure 16, there is an indistinct centrifugally located clear zone. According to Conklin (1931), this is composed of test cells that have not yet been extruded. They have been listed as 6 per cent other granules in the calculations included in Table 1.

Certain centrifuged eggs, showing five or six zones of stratification instead of the customary three, are of particular interest since they possibly indicate that there are two types of hyaline protoplasm of different density. *Nereis* eggs centrifuged with forces of 240,000 times gravity give a very satisfactory picture of these two hyaline zones. The toughness of the egg membrane is advantageous in enabling the *Nereis* egg to withstand the forces necessary to bring about this separation of substances. In certain other cases, lower forces have accomplished a similar separation of materials. Clement (1935, p. 411) has figured an *Ilyanassa* egg showing five zones of separated material, including two hyaline zones. The middle zone, however, is believed by Clement to consist of the mitochondria. In *Urechis*, Taylor (1931) also observed five zones in eggs centrifuged for some hours at a force of 4,800 times gravity. These zones were as follows: the black cap, brown stratum, yellow stratum, hyaline zone, zone of red granules. In my experiments the black cap (oil?) occupied about 7 per cent, the hyaline zone about 49 per cent, the germinal vesicle 9 per cent, and the heme granules about 1 per cent of the egg volume. The remainder of the egg (34 per cent) includes the other granules. Because of the unusual position of the hyaline zone and the absence of a definite zone at the centrifugal pole comparable to the yolk zone of other eggs, it does not seem possible to homologize the zonation of the *Urechis* egg with the zonations of other egg species. For this reason the calculations for the *Urechis* egg have been omitted from Table 1.

The relationship between the substances in the various eggs that are loosely termed "yolk" is unknown. Yolk granules in the different eggs vary in size, color, density, and staining reactions. For the purposes of this paper, however, yolk may be defined as a substance of density greater than that of hyaline zone I, and not brilliantly pigmented. This definition includes all materials listed as yolk in Table 1, and excludes the pigment granules.

Homologies between the lipoid (oil or fat) layers of the several egg species may be more readily ascertained, since tests for lipoid substances are possible. The oil stratum is the layer of lowest density and therefore occupies the most centripetal position. It is recognized that all the fatty or lipoid substances of the egg are not present in the protoplasm as discrete formed droplets, and that the volume of the oil cap does not represent the total lipoid content of the egg.

The photomicrographs of typical centrifuged eggs (Pl. I, Figs 1–16) show a fundamental similarity of stratification. The classification of the strata into the several categories indicated in Table 1 is therefore considered justifiable. A photomicrograph of a centrifuged *Arbacia* egg showing more satisfactory stratification than that of Plate I, Figure 11, is presented by Heilbrunn (1937, Fig. 14). Costello also figures a number of centrifuged *Cumingia* eggs (1934) and of centrifuged *Asterias* eggs (1935). In Plate I the degree of stratification shown is in some cases submaximal. Other figures, showing greater stratification, but less satisfactorily reproducible, were employed for the calculations.

DISCUSSION

The results included in this paper may be compared with those of other investigators in a few instances. Conklin (1916) observed three zones in the centrifuged egg of *Crepidula plana*. These were the oil zone, hyaline zone, and yolk zone, occupying 2 per cent, 22 per cent, and 76 per cent, respectively, of the egg volume. Considering the difference in magnitude of the centrifugal forces employed (Conklin used a centrifugal force of 2,000 times gravity), these values agree fairly well with the volumes of comparable zones in the gastropod eggs studied (i.e., the nudibranchs *Anisodoris*, *Archidoris*, *Cadlina*, *Diaulula*, *Hopkinsia*, and *Rostanga*). The results in Table 1 may be compared with the measurements of E. N. Harvey (1932) and of Holter (1936), in which corrections were made for the volume of hyaline protoplasm between the packed granules. Allowing for this correction, one may conclude that the results obtained by different investigators upon different lots of eggs within the same egg species may agree within about 10 per cent.

In every case (but one) in which comparisons are possible, the volume of the formed components exceeds the volume of the osmotic dead space. For example, while Białaszewicz (1933) found the osmotic dead space of eggs of four species of echinoderms to vary from 19.3 per cent to 36.4 per cent, my calculations of the volume of the dispersed phase for four other species of echinoderm eggs are as follows: *Arbacia*, 55 per cent; *Asterias*, 80 per cent; *Echinarachnius*, 80 per cent; and *Strongylocentrotus purpuratus*, 72 per cent. The application of the maximal correction for the volume of hyaline protoplasm located between the packed granules reduces these figures to 40.7 per cent, 59.2 per cent, 59.2 per cent, and 53.3 per cent, respectively. McCutcheon, Lucké, and Hartline's value (1931) for the osmotically inert material of the *Arbacia* egg (7–14 per cent) is far below the value for the relative volume of the formed components.

Fauré-Fremiet (1924) studied the osmotic properties of the eggs of *Sabellaria alveolata*. Making certain assumptions based upon the water content of the egg, he estimated that 30 per cent of the cell volume consists of "dry substance." The term "dry substance," originally used by Overton, is, according to Lucké and McCutcheon (1932), equivalent to that fraction of the cell volume which does not take part in osmotic exchange of water.

Lucké and McCutcheon (1932) calculated from the data of Ephrussi and Neukomm (1927) that 46 per cent of the cell volume of the unfertilized egg of *Paracentrotus lividus* is occupied by osmotically inactive material. This is the highest figure recorded for a marine egg, and is of the order of magnitude of the volume occupied by the formed components of related egg species.

The "nonsolvent volume" of the eggs of *Echinometra lucunter* was calculated by Leitch (1937). He found that the nonsolvent volume of the eggs remains relatively constant at an average value of 34 per cent of the initial egg volume for almost 2 hours after spawning. On longer standing before use, the value was increased to 53 per cent.

SUMMARY

The relative volumes occupied by the formed components of centrifuged eggs have been calculated for twenty-six egg species. If these relative volumes are not corrected for the volume of hyaloplasm occupying the interstices between the granules, the volume occupied by the formed constituents varies from 40 per cent in the egg of *Hydroides* to 82 per cent in the egg of *Polychoerus*.

The relationship between the relative quantities of formed components in marine egg cells and certain problems of cellular physiology is discussed.

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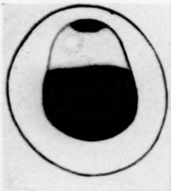
PLATE I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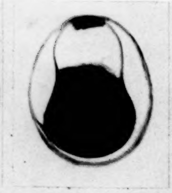
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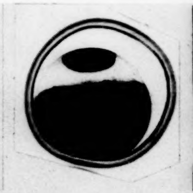
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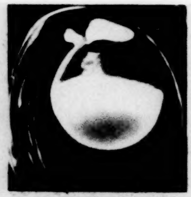
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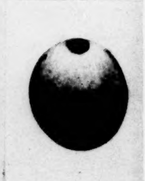
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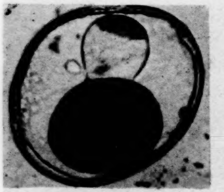
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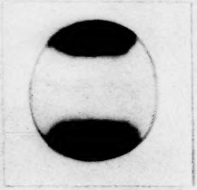
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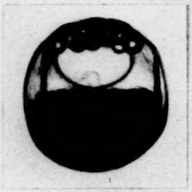
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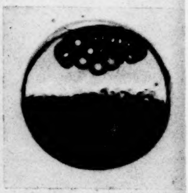
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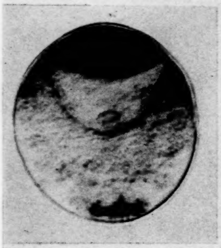
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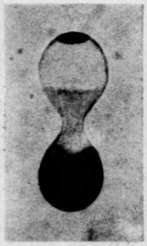
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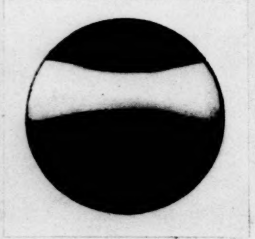
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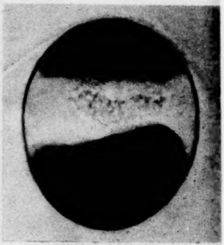
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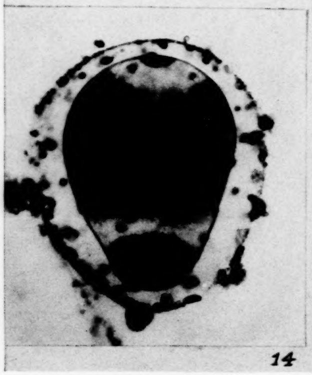
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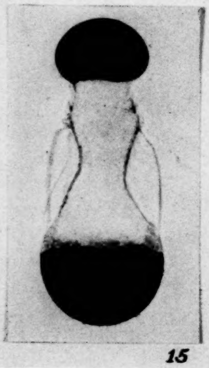
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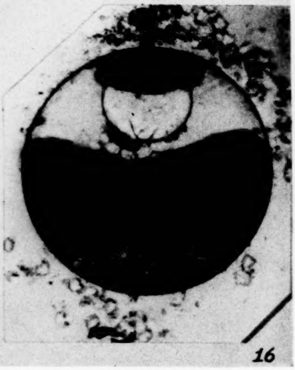
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PLATE I

All figures are photomicrographs of living centrifuged eggs. The centripetal pole is uppermost in each case.

FIG. 1.—*Diaulula* egg centrifuged 50 minutes at about 4,800 × g., at the time of formation of the first polar body; photographed 9 minutes later. The first polar body is represented by the blurred spot in the hyaline segment.

FIG. 2.—*Hopkinsia* egg centrifuged 8 minutes at about 25,000 × g., photographed 6 minutes later.

FIG. 3.—*Rostanga* egg centrifuged 8 minutes at about 26,000 × g., photographed 5 minutes later.

FIG. 4.—*Anisodoris* egg centrifuged 7 minutes at about 27,000 × g., photographed 8 minutes later. Dark-field illumination.

FIG. 5.—Unfertilized *Asterias* egg centrifuged 12 minutes at 6,000 × g., 10 minutes after entering sea water; photographed 2 minutes later.

FIG. 6.—*Archidoris* egg centrifuged 17 minutes at about 28,000 × g., before the formation of the first polar body, photographed 31 minutes after centrifuging. Note polar body at the hyaline segment.

FIG. 7.—Unfertilized *Cumingia* egg centrifuged 10 minutes at 6,000 × g., photographed 9 minutes later.

FIG. 8.—Unfertilized *Nereis* egg centrifuged 60 minutes at 6,000 × g., photographed 8 minutes later.

FIG. 9.—Fertilized *Nereis* egg centrifuged 7 minutes at 6,000 × g., 27 minutes after insemination; photographed 8 minutes after centrifuging.

FIG. 10.—Unfertilized *Urechis* egg centrifuged 55 minutes at 4,800 × g., photographed 12 minutes later.

FIG. 11.—Unfertilized *Arbacia* egg centrifuged 20 minutes at 6,000 × g., photographed 3 minutes later.

FIG. 12.—Unfertilized *Phascolosoma* egg centrifuged 23 minutes at 6,000 × g., photographed 15 minutes after centrifuging.

FIG. 13.—Unfertilized *Sabellaria* egg centrifuged 19 minutes at 6,000 × g., photographed 19 minutes later.

FIG. 14.—Unfertilized *Echinarachnius* egg centrifuged 24 minutes at 6,000 × g., photographed 5 minutes later.

FIG. 15.—Unfertilized *Amphitrite* egg centrifuged 17 minutes at 6,000 × g., photographed 9 minutes later.

FIG. 16.—Unfertilized *Styela* egg centrifuged 10 minutes at 6,000 × g., photographed 14 minutes later. Note oil droplets caught below membrane of the germinal vesicle.

THE CHEMICAL NATURE OF THE AMPHIBIAN ORGANIZER: III. STIMULATION OF THE PRESUMPTIVE EPIDERMIS OF AMBYSTOMA BY MEANS OF CELL EXTRACTS AND CHEMICAL SUBSTANCES[1]

(One plate)

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STUDIES on the chemical nature of the amphibian organizer have become complicated by the fact that so many unrelated substances and extracts will stimulate the formation of a neural plate or neural tube. Fischer and his co-workers (1935) suggest that some property of these seemingly unrelated substances, such as their acidity, is the stimulus for neural tube formation. Waddington *et al.* (1936) have attempted to explain most neural inductions as caused by a sterol present in the substances used, or by a liberation of this sterol within the presumptive epidermis by application of the substances.

TABLE 1

NEUTRAL INDUCTION IN *Ambystoma opacum* BY MEANS OF CEPHALIN

Kaolin used as a carrier and cholesterol in kaolin as control

Substance	Preparation	Operated	Survived	Growth	Neutral Plate	Neutral Tube
Cephalin	A	26	20	7	5	5
Cholesterol	B	20	14	1	0	0

The purpose of these researches was to test out lipid extracts for neural inductor capacity and to try to substitute artificial inductors. When it was found that substances like digitonin, acids, and bases were able to act as artificial neural inductors, it was evident that a reinvestigation of the naturally occurring inductor must be undertaken in light of these new facts. For it then becomes important in using any tissue extract to distinguish between an artificial inductor and the natural inductor in the roof of the archenteron. Therefore, a comparison was made of the inductions obtained by lipid extracts and those obtained from protein residues to see where the greatest potency resided.

In an earlier paper (Barth, 1934) the crude cephalin fraction of mammalian brain was used for neural induction in *Ambystoma*. Later (Barth, 1935) it was found that purified cephalin would induce large neural tubes in *Ambystoma opacum*. In the following experiments cephalin was prepared from calves' brain, according to the methods of Parnas and Renall as outlined in Thierfelder and Klenk (1930). The cephalin obtained was mixed with kaolin to form a paste which could be cut into small pieces and placed in the blastocoel with a pair of sharp forceps. As a control, cholesterol prepared from the brain was mixed with kaolin and implanted in the same way.

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[1] These researches were aided by a grant from the Rockefeller Foundation for research in chemical embryology.

Table 1 records the results. Fifty per cent of the embryos which survived showed neural induction, and an example is shown in Plate I, Figure 1, where a large irregular tube-resembling brain is induced in the ventral body region. Posterior to the section shown in Figure 1, vesicles are present which are interpreted as otic vesicles. There are no connections between the primary neural tube of the host and the induced structures.

NATURE OF THE ACTION OF CEPHALIN

It is important to know what property of cephalin is responsible for the neural induction, and the next series of experiments were performed to throw some light on its action. Needham, Waddington, and Needham (1934) reported that a substance precipitated by digitonin caused neural induction in *Triton*, and Fischer, Wehmeier, Lehman, Jühling, and Hultzsch (1935) showed that various substances, regardless of their chemical nature, induced neural plates when rendered acid by shaking with acids.

TABLE 2

THE ACTION OF VARIOUS PREPARATIONS OF CEPHALIN, THE PRODUCTS OF HYDROLYSIS OF CEPHALIN, AND STEROL FROM BRAIN ON THE PRESUMPTIVE EPIDERMIS OF *Ambystoma opacum* IMPLANTED INTO BLASTOCOEL

Preparation	Concentration (Per Cent)	Operated	Survived	Growth	Neural Plate	Neural Tube
C (hydrolyzed cephalin)	25.0	30	11	....	1	2
D (see text)	....	70	32	3	4	2
E (sterol)	100.0	44	32	0	1	0
F (cephalin)	9.0	16	12	5	1	0
G (cephalin)	3.0	16	13	....	1	0
H (cephalin)	14.0	22	14	....	0	0
I (cephalin)	10.0	91	34	32	0	1
J (cephalin)	5.0	24	16	0	0	0
K (cephalin)	0.5	74	12	0	0	0
L (cephalin)	....	24	13	0	0	0

Accordingly, cephalin was hydrolyzed, and the products of hydrolysis tested and found to be active (Table 2, preparation C, and Fig. 2). Next a sample of cephalin was saponified with 2 N alcoholic potassium hydroxide. After acidification and extraction with ether, the products of hydrolysis were dissolved in warm 95 per cent alcohol and 1 per cent digitonin in 95 per cent alcohol added. No precipitate formed, but, upon cooling 24 hours at 5° C., the solution became cloudy and was centrifuged. The supernatent liquid was evaporated to dryness, and this residue extracted with petroleum ether. This preparation (D, Table 2), was tested and found to be active. Thus the action of cephalin and the product of hydrolysis of cephalin as a neural inductor are not due to the presence of an impurity in the form of a sterol which precipitates with digitonin such as Needham *et al.* found in the ether extracts from glycogen.

The sterol fraction was tested directly in the following manner. Two calves' brains were extracted with acetone twice, alcohol once, and ether twice, and the united extracts evaporated to remove solvents. A portion was saponified for 5 hours with 2 N alcoholic potassium hydroxide, and, after acidification and extraction with ether, the products of hydrolysis were dissolved in warm 95 per cent alcohol and 1 per cent digitonin in

95 per cent alcohol added, and kept overnight at 5° C. A voluminous precipitate was obtained, and the filtrate tested with digitonin until no more precipitate formed.

The sterols were recovered from their digitonides by means of anhydrous pyridine followed by precipitation with petroleum ether. This preparation (E) was very weak in its action, forming a neural plate but no tubes. It seems likely from this result and the foregoing experiments with preparation D that the action of cephalin from the brain is not due to a sterol impurity as suggested by Waddington, Needham, Nowinski, Lemberg, and Cohen (1936).

Since Fischer *et al.* (1935) found that simple acidification of substances caused them to become neural inductors, this possibility was tested in the case of cephalin. Using the method of extraction as outlined in Thierfelder and Klenk (1930), one of the steps is to precipitate the cephalin with HCl, thus forming an acid. The cephalin is then washed with acetone and dried *in vacuo*. Subsequent treatment merely involves preciptation with acetone from ether solutions. The cephalin thus prepared is an acid, and its action as a neural inductor may be due to the acid stimulus of Fischer *et al.*

Several observations indicated that cephalin (preparation A) was toxic. In the first place, after it was inserted in the blastocoel, the cells with which it made contact began to cytolyze, and this action sometimes spread until the whole embryo cytolyzed. Second, in some experiments the preparation was placed on the surface of the early gastrula in contact with the vitelline membrane. Something diffused through the membrane and killed the cells underneath. This action could be observed directly under the binocular microscope. The dead cells formed a layer which lifted off from the living cells. The living cells underneath this dead layer became pigmented and folded, suggesting neural induction resulting from the dead layer. This has not yet been confirmed in sections of the embryos. Finally, the preparation proved to be acid to litmus.

In addition to the foregoing observations, further experiments with different preparations of cephalin in varying concentration gave variable results, some preparations giving induction, others not (see Table 2, preparations F–L). Of special interest in this table is preparation I, which is cephalin which was kept under acetone for a year. Its action is remarkably constant, always stimulating large outgrowths with a few elongate nuclei but never neural plates or tubes (Fig. 3).

A similar preparation of cephalin was implanted in *A. mexicanum* (Table 3, preparation I). Here, too, large outgrowths result as in Figure 3. In this form, as in *A. opacum*, the effect of cephalin was variable, one preparation showing induction, another not (Table 3, I, M, X).

Finally, in *A. punctatum* the results are much the same as in the other species (Table 4). Preparation A, which is very effective in *A. opacum*, has not been tried, and most of the other preparations of cephalin failed to induce. Preparation I, however, stimulates large outgrowths which for the most part show little or no neural induction. Other preparations may occasionally form large growths but not so consistently as preparation I.

Here (Table 4) are also included some tests with synthetic cephalin prepared by two students, Mr. Leonard C. Miller and Mr. James S. Missett. Some of the intermediate products were also tried, such as α and β distearin and the di-stearchlorhydrins from these. The latter induced a small neural tube.

Other fractions of calves' brain proved ineffective. Crude lecithin (preparation Q) and crude sterol fraction (preparation R) were negative. Preparation E, the sterol recovered after precipitation by digitonin, also gave no action. This lack of induction in *A. puncta-*

tum with various lipid extracts led to the use of the living dorsal lip of the blastopore as an inductor in order to see whether the epidermis would react under the conditions of the experiment. In these experiments the living dorsal lip was wrapped up in jackets of presumptive epidermis, and good neural induction was obtained. In a series where the dorsal lip was killed with alcohol or dried at room temperature no induction was found, and the dead implants caused cytolysis of the presumptive epidermis in which they were placed. It seems quite clear that the epidermis of *A. punctatum* is not easily stimulated by dead implants or extracts but that it will react to the living organizer. It should form, therefore, a good test object for the naturally occurring organizer.

TABLE 3

EFFECT OF CEPHALIN IMPLANTED INTO THE BLASTOCOEL OF *Ambystoma mexicanum*

Preparation	Concentration	Operated	Survived	Growth	Neural Plate	Neural Tube
I	5 per cent in agar	22	21	16	0	0
M	100 per cent	23	13	....	2	0
X	100 per cent	33	31	....	0	0

TABLE 4

EFFECT OF CEPHALIN AND OTHER EXTRACTS OF BRAIN IMPLANTED INTO BLASTOCOEL OF *Ambystoma punctatum*

Preparation	Concentration	Operated	Survived	Growth	Neural Plate	Neural Tube
I (cephalin)	5.0 per cent in agar	38	30	18	1	0
I (cephalin)	0.5 per cent in agar	90	31	6	0	0
I (C)	0.5 per cent in lard	15	9	6	0	0
N (synthetic cephalin)	5.0 per cent in agar	30	17	....	0	0
O (distearin)	5.0 per cent in agar	15	12	....	0	0
P (distearchlor-hydrin)	5.0 per cent in agar	45	18	....	0	1
Q (crude lecithin)	5.0 per cent in agar	26	24	....	0	0
R (crude sterol)	5.0 per cent in agar	23	12	....	0	0
E (sterol)	2.5 per cent in agar	25	18	....	0	0
S (cephalin)	50.0 per cent in kaolin	60	45	....	0	0

A number of extracts were tried in a large number of cases in one or more of the three foregoing species of *Ambystoma* with no signs of induction. These are sphingomyelin, crystalline preparations of the cerebrosides, glycerophosphoric acid, and lecithin.

THE ACTION OF TOXIC AGENTS ON THE PRESUMPTIVE EPIDERMIS

The results with cephalin suggested that it was acting as a neural inductor by virtue of its toxicity and probably its acidity. Only in high concentrations did cephalin act, and even then some samples did not induce. Since the entire ectoderm of the gastrula possesses the ability to induce after being killed in various ways (Holtfreter, 1934), it seemed possible that cephalin, by killing or injuring some of the cells with which it came in contact, caused these killed or injured cells to act as inductors.

Another and more likely possibility is that the formation of the neural tube is a reaction to a stimulus much as an egg reacts to parthenogenetic agents. Many toxic agents, if applied for a short time only, will activate an egg without killing. The action of cephalin, then, may well be a direct stimulus to the cells of the presumptive epidermis, which respond by forming a neural tube. It is not necessary to assume, as have Waddington *et al.* (1936), that foreign substances such as methylene blue act by release of the neural inductor. These substances may act directly by substituting for the normal stimulus of the organizer. There is no evidence that the neural inductor is present in the presumptive epidermis, since all that the experiments show is that, after killing, the dead cells will stimulate a neural tube. It is significant that any source of dead cells will act (Holtfreter, 1934). Since dead cells liberate all sorts of toxic substances which might act as stimulating agents, just as methylene blue and cephalin, and, indeed, the sterol suggested by Needham *et al.* (1934; Waddington *et al.*, 1936) as the naturally occurring inductor, there is as yet no evidence that these dead cells contain the naturally occurring inductor.

TABLE 5

EFFECT OF DIGITONIN ON THE PRESUMPTIVE EPIDERMIS OF *Ambystoma mexicanum*

Neural induction as in Figs. 4 and 5. Useful cases are those in which the implant is in contact with the epidermis. Concentration is percentage by weight in powdered egg albumen or in lanoline.

Concentration of Digitonin (Per Cent)	Useful Cases	Neural Induction as in Figs. 4 and 5	Percentage
10.0	6	4	66
1.0	9+2*	5+1	55
0.5	15	12	80
0.1	10	8	80
0.05	7	5	70
Total cases	47+2	34+1	70

* Two cases of explants of presumptive epidermis, one of which gave typical reaction.

Certainly, the wide variety of substances and tissues which stimulate the presumptive epidermis to form a neural tube do not suggest that we are dealing with a naturally occurring inductor any more than the wide variety of parthenogenetic agents throw any light on the naturally occurring stimulus in the sperm.

In any event foreign substances applied to the epidermis *do* cause neural tubes to form, and so other substances known to be toxic to cells were tried. Digitonin was used because it was possible that in some of the earlier work of Needham *et al.* (1934) the action of the sterol may have been due to the digitonin which was not separated from it.

Digitonin was therefore mixed in varying concentrations with lanoline and also with powdered egg albumen as a carrier and these mixtures implanted into the blastocoel of *A. mexicanum*. The reaction is remarkably constant when the implant comes in contact with the presumptive epidermis. The cells are converted into a small plate resembling the cells in the neural tube very closely (Figs. 4 and 5). Table 5 records the results with varying concentrations. It is seen that digitonin acts in low concentrations, and the lowest tried, which was 0.05 per cent, was still active. Similar results were obtained with *A. punctatum* where, out of 18 useful cases, 5 showed the typical digitonin reaction (Figs. 4 and 5).

USE OF OTHER TOXIC AGENTS

The fact that cephalin acted as an inducing agent in toxic concentrations in *A. opacum* and that digitonin caused a similar reaction in *A. mexicanum* and *A. punctatum* suggested that other toxic agents might stimulate. Since Fischer *et al.* had already shown that fatty acids and acid nucleoprotein, etc., would induce, it seemed probable that H-ion concentrations outside the physiological range might also stimulate. Using powdered egg albumen as a carrier, buffers at various pH were mixed with the albumen and inserted into the blastocoel. This method does not give the pH of the egg albumen in contact with the epidermis, and the pH indicated in this paper is merely that of the original phosphate or borate buffer before mixing with the egg albumen.

TABLE 6

THE ACTION OF VARIOUS BUFFETS UPON THE PRESUMPTIVE EPIDERMIS OF *Ambystoma mexicanum* AND *A. punctatum*

Reaction as in Figs. 4 and 5. Useful cases are those in which the implant is in contact with the epidermis. Phosphate or borate buffers mixed with powdered egg albumen as a carrier.

pH of Buffer	Total Cases	Useful Cases	Neural Induction
3.0	36	12	7
4.0	21	10	0
5.0	15	6	1
Egg albumen control	18	10	0
8.1	17	6	1
8.7	13	11	0
9.7	39	22	7

Table 6 shows that the most effective buffers are those at pH 3.0 and 9.7—the extremes of the range tested. The numbers are admittedly small, but the main point here is that both acid and alkaline buffers stimulate. In comparing the effect of buffers with digitonin, it should be pointed out that the reaction with digitonin is more constant and a little more extensive.

THE QUESTION OF THE NATURALLY OCCURRING INDUCTORS

In view of the results presented in this paper and those of Fischer *et al.*, considerable doubt as to the lipid nature of the neural inductor is aroused. If foreign substances introduced into the blastocoel produce changes in the cells similar to those produced by the neural inductor of the organizer, it is extremely difficult to know when one is working with the natural occurring inductor. Careful quantitative and qualitative studies on the response of the ectoderm to the chemical stimulus in various concentrations will help; but, finally, the substance claimed as the naturally occurring inductor must be shown to be present in the roof of the archenteron.

Certainly, if the inductions described in this paper and also in the papers of Needham *et al.* and Waddington *et al.* (1936) are compared with those obtained by the natural organizer, one is immediately struck by the low percentage of induction and the low degree of differentiation of the chemically induced neural tubes. The lack of mesodermal structures and sensory structures is conspicuous. Further, a comparison of Holtfreter's inductions with protein residues after ether and alcohol extraction with Needham's inductions with sterol shows clearly that the greater potency is in the protein residue. Both of these investigators used the same test object, *T. taeniatus*.

Accordingly, a series of experiments with fat-free calves' brain and frogs' gastrula have been started in order to compare induction with various lipids. The fat-free fraction of calves' brain was prepared by drying in two changes of acetone, followed by extraction with alcohol and two extractions with ethyl ether. A sample of the finely divided residue was then treated with boiling 95 per cent alcohol for 11 hours and filtered hot. It was further treated with boiling ether filtered and refluxed once more with 95 per cent alcohol for 3 hours, filtered, and the process repeated. The residue was finally boiled with ether and filtered.

This residue gave very good induction of neural tubes in 80 per cent of the cases when implanted in *A. opacum* (Fig. 6). In ten cases where the implant came in contact with the presumptive epidermis, eight showed good neural induction. There may possibly be some either soluble substances left in this residue, and so this possibility was tested in the following way.

After preliminary extraction of the brain with acetone, alcohol, and ether some of the residue was treated with pepsin HCl for several days, after which the mixture was extracted with ethyl ether. The ether was removed, and the lipid picked up with petrol ether. This lipid extract was tested in 10 per cent, 1 per cent, and 0.1 per cent concentration in egg albumen in *A. mexicanum*. In a few cases a reaction of the epidermis was obtained which was identical with that produced by digitonin and acid buffers at pH 3. No neural tubes were obtained. This lipid is not the active principle of the fat-free residue. However, there may still be protein lipid or glycogen lipid combination not broken up by pepsin-HCl or extracted by prolonged fat solvents, and this possibility must be tested by other methods of hydrolysis of the proteins.

DISCUSSION

Recent reviews (Weiss, 1935; Woerdeman, 1936) have pointed out the difference in the action of the living organizer and the action of dead organizer and chemical substances. In the first place, living organizer loses its specificity for inducing particular structures when killed by various means (Holtfreter, 1934). All parts of the organizer after killing behave like any source of dead tissue. Second, when attempts are made to extract active principles, they give only neural tubes, and the associated structures like the sensory structures are lacking. Finally, when relatively pure substances are used, only small plates or tubes are elicited. In each of these steps from living organizer to pure substances something is lost, and a satisfactory chemical preparation to be used as organizer is still wanting. From a comparison of protein residues and lipid extracts, it appears that most of the potency remains in the residue.

It is suggested therefore that the protein residue may contain the naturally occurring organizer and that the lipid extract merely acts as does digitonin, di-benzanthracene, and other artificial stimulators. It is highly significant in this respect that all Holtfreter's treatments of the organizer—heat, drying, HCl, etc.—lead to a denaturization of the proteins with the result that the specificity of induction is lost.

Induction of neural structures is, then, the result of a general stimulus applied to the presumptive epidermis. The inductors in the organization center, however, have a specific effect which has been lost in all attempts to extract the substances responsible for this specific action. The reactivity of the presumptive epidermis includes formation of all the sensory structures, notochord, mesodermal somites, pronephros, and other structures. None of these structures has as yet appeared as a result of chemical stimulation, and only rarely have they appeared with dead organizer.

The fact that digitonin, acids, bases, and other substances producing cytolysis act as neural inductors recalls the case of artificial parthenogenesis in the sea urchin, where similar agents stimulate development. In both cases the stimulus is of a general nature acting on a preformed mechanism which is called into action by a variety of substances.

There are apparently two methods by which a neural tube may be stimulated to form from presumptive epidermis: first, by a variety of dead tissues and chemical agents which may be classified as artificial inductors and, second, by some specific substance or substances in the organizer which are natural inductors. The nature of these substances is not known, but my experiments suggest that they are proteins.

SUMMARY

1. Cephalin acts as an inductor of neural tubes in *A. opacum* only when it is somewhat toxic because of its acid properties.

2. Other toxic agents such as digitonin, acid, and alkaline buffers will stimulate the formation of a neural plate in *A. mexicanum* and in *A. punctatum*.

3. A comparison of protein residues and lipid extract shows the potency for neural induction to be greater in the protein residue.

4. The suggestion is made that substances previously used as inductors are artificial inductors and that the naturally occurring inductors are probably proteins.

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PLATE I

Induction of neural structures in the epidermis of *Ambystoma*. All figures show the induced neural plate or tube in the ventral body region, and the induced structures have no connection with the host neural tube. The implant in all cases is adjacent to the induced structure.

FIG. 1.—Induction of neural tube in *Ambystoma opacum* by means of cephalin. Preparation M used.

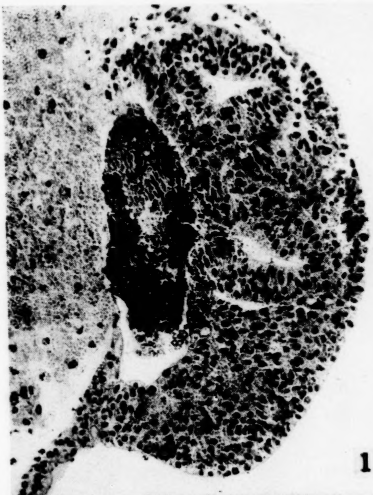
FIG. 2.—Induction of neural plate and folds in *A. opacum* by means of the products of hydrolysis of cephalin.

FIG. 3.—Large epidermal proliferations induced by cephalin in *A. opacum*. Preparation I used.

FIGS. 4 and 5.—Inductions of neural plates in *A. mexicanum* by means of digitonin.

FIG. 6.—A neural tube induced in *A. opacum* by the protein residue after extraction of lipids.

PLATE I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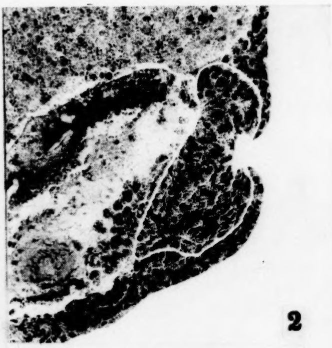
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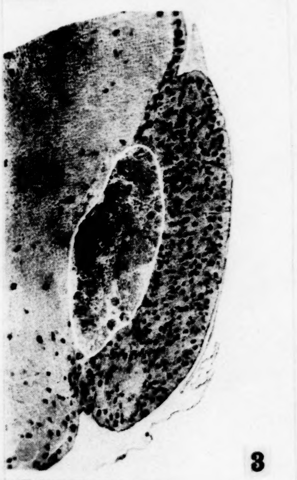
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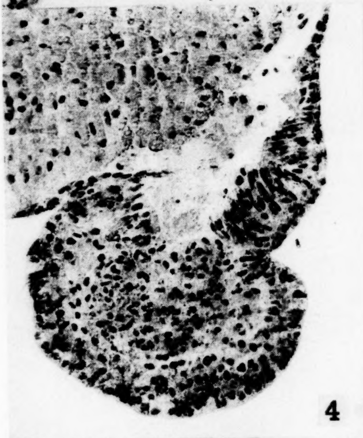
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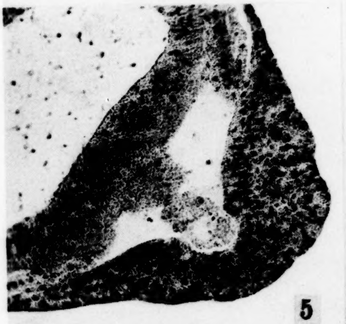
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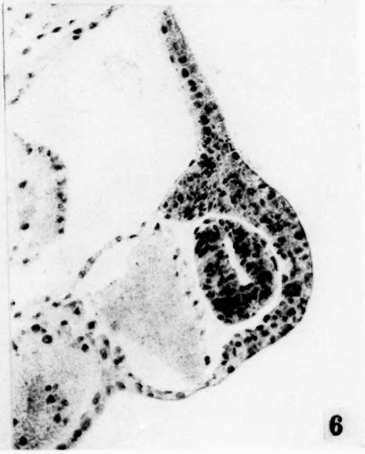
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UNIV. OF MICH.

STUDIES ON THE ANTAGONISM BETWEEN IMPLANT AND HOST IN FISH EMBRYOS

(Two plates)

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WHILE studying the action of certain drugs on the innervated and denervated fish heart (Brinley, 1935), it was found desirable to ascertain the effect of certain drugs on a pulsating heart which had been excised from its normal blood supply and nerve connections and implanted into the side of the yolk sac in another individual in such a position that it could be easily observed and would not likely be innervated by nerves from the host. These implants developed and continued beating for a maximum period of 12 days. At the end of that time, the rate and amplitude of the host heart gradually decreased until finally death occurred prior to stoppage of the contractions of the implant. This was a very surprising result, and a search of the literature revealed no exact parallel case. So it was deemed advisable to restudy the problem in detail to see if more light could be thrown on the general problem of the incompatibility between host and implant.

The great majority of the work on organ transplantation has been done on amphibians, with some work on birds and mammals, but little has been reported on fish (Koppanyi, 1923; Blatt, 1924; Mangold, 1931). In the work here reported we have been especially interested in studying the physiological action of different types of implanted tissue on the host, using *Fundulus heteroclitus* as the donor and *F. majalis* or hybrids between the two species as recipients, and homoplastic transplants with brown trout (*Salmo fario*) and the hardhead minnow (*Hepsetia stipes*). Three types of tissue were selected for the experiment: (1) the heart, as representing a large, well-organized and actively functioning structure; (2) the eye, also large and well organized, but passive, with activity apparently limited to growth changes; and (3) glandular tissues, the liver and spleen, with the thought that they would present an intermediate type of activity, not being as well organized as the others and falling somewhere between them in functional activity.

HEART

In all the experiments reported on the heart unless otherwise stated *F. heteroclitus* was used as the donor and *F. majalis* as the recipient. The embryonic heart develops early and begins to pulsate at room temperature on the third to fourth day after fertilization. It is relatively large and stands out in plain view, upon the anterior surface of the yolk sac. It beats forcibly and regularly at a uniform rate of about 130–40 beats per minute at temperatures of 20°–22° C.

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In preparing the heart for implantation, *F. heteroclitus* embryos from 5–10 days after fertilization were placed in 60 per cent sea water (60 parts sea water, 40 parts distilled water), which is nearly isotonic with the excised heart. The chorions were removed with fine dissecting scissors, and the naked embryos floated out in the dilute sea water. This operation does not injure the embryos, as is evidenced by their ability to develop normally. The heart, which consists of a single auricle and ventricle, can be seen in its normal position and is covered by a thin membrane, the pericardium, which seems to form a part of, or unites with, the membranes of the yolk sac. After removal of the chorion, the pericardium is cut with a fine needle scalpel and carefully pulled away from the anterior portion of the yolk sac, leaving the normally beating heart exposed but still connected to the host blood vessels. The aorta is then cut where it leaves the heart, and, by gently elevating and pulling the tip of the ventricle, the heart can be lifted away from the yolk sac and finally severed by cutting the veins. The excised heart will continue to beat in isotonic sea water for 3 or 4 hours. Implantation may be made at any time during this period, but the best results are obtained if the transfer into the host is made as soon as possible after the heart is taken from the donor. In all cases here reported the entire heart (auricle and ventricle with the cut ends of the arteries and veins) was used as the implant; but, if the heart is divided at the auriculoventricular junction, either portion will continue to beat when implanted into the host. The auricle will soon resume contractions at the normal rate, but the isolated ventricle always beats much slower, often only a few beats per minute.

In preparing the embryos of *F. majalis* to receive the implant, the chorions are removed from embryos 8–10 days after fertilization, at least a day before implantation, and only embryos are used which are in good condition with strong, well-established circulations. The naked embryos are then placed in isotonic sea water, and a very small slit is made through the membranes where the yolk sac joins the embryo. The cut is made just back of the large vessels which carry blood to the yolk.

The pulsating heart, previously prepared, is quickly transferred with teasing needles to the slit made in the yolk sac and held in place for a few seconds until the membranes close around the implant and hold it in place. The implant must not be pushed into the yolk so that it will be submerged when the cut in the membrane is repaired. For a more detailed report of the operative technique the reader is referred to a previous paper (Brinley, 1935).

In the majority of cases the implant did not stop beating while it was being removed from the donor and transplanted into the host. The rate of contraction slowed considerably for the first few hours after the operation but gradually increased until the fourth or fifth day, when the normal rate was reached and maintained. The implant pulsates as rapidly as the host heart but does not synchronize with it. Usually both chambers of the heart pulsate at a one to one ratio, but at other times only the auricle contracts; or both chambers may contract independently of each other. The inclosed blood corpuscles, carried over in the implant at the time of operation, can be seen to ebb and flow as the heart contracts. In only a few cases did the host blood vessels make contact with the implant. In one case a host capillary grew into the walls of the implant, and in one other case a host capillary entered and carried blood into the lumen of the implant, and the blood was forced back into the capillary at each contraction of the heart. In numerous cases host capillaries developed in the membranes surrounding the implant.

The average length of life of the implant and host was 5–6 days, but some of them lived a maximum of 12 days. The initial effect of the implant on the host was found to consist of a slowing of the ventricular beat which rapidly passed to complete stoppage. Slight contractions of the auricle continued for several minutes, and finally the entire heart stopped in diastole within 3–4 hours after the first effect was noticed. In practically all cases the death of the host preceeded by a few hours the death of the implant. In these cases the beating of the implant could be observed, after the heart and circulation of the host had stopped. This reaction was seen when both *F. majalis* and the hybrids were used as hosts. If the implant did not "take," the shriveled remains appeared as a white coagulum on the surface of the yolk sac and did not interfere with the normal activity of the host. Therefore, death of the host is evidently due not to the presence of foreign tissue of protein but probably to the added amount of waste material or to some hormone or humor produced by the actively beating implant which is antagonistic to the heart of the host.

Histological studies of sectioned material show that the implant developed between the ectoderm and mesoderm or between the mesoderm and entoderm. The ectoderm adjacent to the implant shows considerable thickening, which appears to be due to a healing-over and a piling-up of cells and not to multiplication, as only a few of these cells were observed undergoing division. Numerous host capillaries could be seen in the mesoderm covering the implant. In several experiments the host membranes did not grow over the implant, and in these cases the implant grew better than when placed under the ectoderm. The cardiac cells grew out over the adjacent ectoderm and formed large globular cells. In the living condition these cells appeared as minute bunches of grapes which sloughed off or could be picked off with dissecting needles (see Brinley, 1935). The implant retains its identity, and the cells are large with prominent nuclei. The ventricle remains as a thick-walled, compact mass of cells. The auricle tends to spread out in a thin sheet of loosely connected cells. Mitotic figures were observed in both myocardial and endocardial cells (Pl. I, Fig. 1), which indicates that there is considerable cell multiplication and growth of the implant. On the whole, there was surprisingly little change in the histological picture of either the cardiac cells or the contained blood cells. These implanted hearts behaved quite uniformly as they would have done in their natural relations, undergoing little change even after 10 or 12 days. The interesting development in this work was the death of the host prior to stoppage of the contractions of the implant. This point will be discussed later.

The ectoderm overlying the implant always shows marked changes. There is some proliferation, but the most striking occurrence was the change in shape of the surface cells, which become elongated and club-shaped with vesicular nuclei in the distal ends. No other host tissue changes were noted.

EYE

The eye of *F. heteroclitus* differentiates rapidly, and, at the end of the third day, the optic cup is well developed and the lens has formed. Within 5 or 6 days pigment develops in the retina and choroid, and the eye gradually becomes darker and glassy in appearance. Heteroplastic implants were made between *F. heretoclitus*, as the donor, and *F. majalis*, the recipient. The chorions were removed and embryos prepared for operation as described in the previous section. The entire eyeball was carefully removed from the donor, at any time after the third day, so as not to puncture the membranes and allow the humor to escape. In the first series of experiments the entire eye was placed in a small slit made in the pericardium of the host anterior to the yolk sac. This can easily be done without injury to the host or without danger of puncturing the eye with resulting loss of humor. The implant was oriented so that the lens faced outward. The implant developed normally, and pigment appeared in the retina and choroid at the same time as in the controls. The implant was pulled ventrally by the pericardium as the yolk of the host was consumed until finally the implant takes a position adjacent to, and in front of, the heart. In some cases the pericardium did not completely surround the implant, and the eye could clearly be observed with the lens projecting from the membrane. The implant at this stage appears quite normal but considerably smaller than the eyes of the controls.

A histological study of the implanted eyes shows no decided change in the retina or lens; the optic nerve regenerated and grew from the eye into any adjacent tissue, such as the myocardium, liver, striated muscle (Figs. 2, 3, 4), or, in one case where the implant was placed next to the normal eye of the host, the regenerated optic nerve followed the contour of the normal eyeball and entered the brain.

As the implant grew and developed in the pericardial cavity, it often crowded the liver or heart out of place, but there seemed to be no other effect upon the host. The only effect noted on the implant was some distortion due to pressure exerted by the surrounding tissues. In some individuals the pericardium grew over and completely surrounded the implant, and in these experiments the eye was gradually absorbed as indicated by a reduction in the size of the eyeball, disappearance of the optic nerve and lens, and, finally, after considerable time, by a complete disappearance of the pigmented layers of the retina and choroid.

Owing to the difficulty of observing the implant in the ventral position, when the host begins to swim, and absorption of the eye when covered by the pericardium, a second series of experiments was conducted by implanting the eye in the yolk sac, in the same location as the implanted heart (i.e., upon the yolk sac adjacent to the embryo). In this position the implant frequently developed between the mesoderm and ectoderm, many capillaries entered the eyeball, and the implant became pigmented and developed normally (Fig. 5). There seemed to be no effect on the host, and no absorption of the implant had occurred when fixed at the end of a month.

In order to obtain more data on the growth of the optic nerve and its possible connection with the nervous system as indicated in the foregoing experiments, a third series of experiments was conducted by placing the implanted eye between the two normal eyes of the host, so that the regenerating optic nerve would be in a position to connect with nervous tissue. For these experiments, homoplastic implantations were made on brown trout embryos obtained from a Minnesota state fish hatchery and kept in finger bowls in a refrigerator maintained at 10° C. (Brinley, 1937). When the circulation was well established and the eyes began to show pigmentation, the chorions were removed from the embryos, and an eye of the donor was carefully removed in isotonic salt solution and placed in a slit cut in the developing skull of the recipient dorsal to the forebrain between the two normal eyes (Pl. II, Fig. 6). In order to facilitate the operation and to keep the host quiet until the implant was firmly established, it was necessary to anesthetize the host. This was done by placing it in a saturated solution of chloroform. When body movements ceased, the embryo was transferred to a small dissecting tray constructed by partly filling a Syracuse watch glass with melted paraffin and cutting a small circular hole in the paraffin, sufficiently large to receive the yolk sac of the embryo. The head and tail of the embryo were allowed to rest in a groove in the paraffin. A longitudinal incision was made with fine dissecting needles through the skull at the base of the forebrain and the implant inserted. The embryo was then returned to the refrigerator, and, by the time it recovered from the influence of the anesthetic, the incision was sufficiently well healed to hold the implant in place so that it was not dislodged by swimming movements of the host. Many of these implants "took well" and developed normally until the hosts were fixed for sectioning at the end of 30–120 days. The implant always remained smaller than the normal eye, capillary circulation was established, but no movement of the implant was noticed. Histological study of the implant was made after fixation in Bouin's solution and staining with Delafield's haemotoxylin. A rather superficial examination indicated normal development. A layer of ectoderm of the host grew over the implant, the lens and retina seemed normally developed, but no comparative cell counts were made. The optic nerve regenerated from the implant and grew toward the brain; usually it did not actually enter the nervous system but wandered along its surface. In one very interesting case, the optic nerve split into three branches after leaving the eye. One branch grew anteriorly, ending in a mass of loose nerve tissue adjacent to the implanted eye. A second branch followed a capillary blood vessel through the fibrous covering of the brain and apparently ended in the region of the nerve cells of the prosencephalon. The third branch grew caudalward alongside the brain; its end relations could not be determined (Figs. 7, 8). It is doubtful if the second branch could have passed through the fibrous covering of the brain if it had not followed the blood capillary. In another interesting case the path of the nerve was obstructed by cartilage, and the nerve grew around the cartilage and entered the brain (Fig. 9).

The implanted eye seemed to have very little effect upon the host as was evidenced by normal development until the host was fixed for study at the end of 30 days in some *Fundulus* experiments or at the end of 80 days in the case of the trout.

In order to obtain further data on the effect of the implant upon the mortality of the host, a series of experiments was conducted, using glandular tissue as the implant, as it seemed probable that gland tissue would occupy an intermediate position between the very active heart and the as yet nonfunctional, hence passive, eye. The liver and spleen were chosen for these experiments because they develop rather early and can be quite easily removed from the living embryo. These studies were made at the Carnegie Laboratory at Dry Tortugas, and the fish used was the hardhead minnow, *H. stipes* (identified by Dr. S. F. Hildebrand). These minnows spawn during the summer months, from the middle of June to September. The eggs are about a millimeter in diameter and may be obtained either by stripping the female or the adults may be kept in specially constructed "live boxes" containing false bottoms in which cheesecloth trays are placed to receive the eggs when they are naturally spawned and fertilized during the night. Long adhesive threads which are attached to the eggs hold them in the trays. The trays can be transferred to running-water aquariums, and the eggs removed as needed. The eggs hatch in 8–9 days. Owing to some undetermined cause, the mortality of these young fish is very high, even under the most favorable culture conditions, but a sufficient number survived to enable us to make some observations which are included here. The pale-green gall bladder can be distinctly seen in the living embryo on the fourth day and can be used as a landmark in locating the liver. The spleen is identifiable at about the same time as a faintly pink oval body which becomes bright red in a day or two. These glands can be easily removed in isotonic sea water, and the entire gland (exclusive of the gall bladder) can be inserted into the yolk sac of the host. In these experiments the liver or spleen was removed from the host embryo from 6–8 days after fertilization and homoplastic implants made into embryos of the same age. Owing to the high mortality in these embryos, experiments and controls, our experiments were not as successful as we had hoped for. Out of a total of 89 liver implantations only 5 went on to any considerable degree of development. In the majority of the cases where the host survived for a sufficient length of time, the implanted liver was rapidly absorbed, often within 24 hours, usually within 2 days. In one of the cases that survived, the implant was placed alongside the liver of the host, and the two livers were completely fused when fixed after 48 hours (Fig. 10). The blood vessels of host and implanted liver appeared to connect up, and the line of fusion was indistinguishable. All five of these animals were active and apparently normal up to the time of fixation. In another experiment all except a very small portion of the implant was absorbed at the end of 24 hours. In the three remaining cases the implants had become well established between the mesoderm and entoderm of the host, and a few small capillaries entered the implant from a large blood vessel in the mesoderm. A few cells showed mitotic figures, and the implants appeared normal in all respects (Fig. 11).

Ninety-seven spleen implantations were made as already described. At the time of operation the spleens were well formed and bright red, due to the inclosed erythrocytes. The glands were handled carefully to avoid injury and the consequent escape of the blood cells. Owing to the high mortality, only a few of the experimental animals developed sufficiently to be of much value. In nearly all cases the implanted spleen rapidly faded in color so that at the end of 2 or 3 days the implant was not visible in the living host. In three sectioned specimens the implant was plainly visible and filled with erythrocytes at the end of 72 hours. In two cases the implant was very distinct, imbedded in connective tissue anterior to the liver (Fig. 12). These implants were well developed and somewhat smaller than the normal host glands. The hosts were actively swimming and apparently normal at the time of fixation. In one case the implant appeared as a bud on the side of the yolk sac. Histological study showed it to be encapsulated by ectoderm. It was much smaller than the normal spleen, but the inclosed erythrocytes showed no evidences of fragmentation or other abnormal changes.

As far as could be determined in such a short period of time and with only these few cases, there was no apparent effect on the activity or behavior of the host, and histological sections showed no host abnormalities.

CONCLUSIONS AND SUMMARY

The experiments presented here have dealt with 38 successful implantations of the embryonic hearts of *F. heteroclitus* to *F. majalis*. Thirty homoplastic eye implants were made with *F. heteroclitus;* 4 heteroplastic eye implants from *F. heteroclitus* to *F. majalis* and an equal number from *F. heteroclitus* to *F. majalis–F. heteroclitus* hybrids. Some 30 homoplastic eye implants were made with brown trout (*S. fario*). In the glandular series 89 implants of the liver and 7 of the spleen were made, using the minnow *Hepsetia;* but, owing to the excessively high mortality in both controls and experiments, only 11 of the experimental animals—5 liver and 6 spleen—lived long enough to present evidence that we considered to be of any value.

In every case the experimental animals were studied daily as a check on the behavior of both host and implanted tissues in the living state and at intervals individuals were killed in Bouin's solution sectioned 10 μ thick and stained with Delafield's haemotoxylin and eosin and studied histologically.

A great majority of the heart experiments were successful, the host surviving for a number of days, the implant beating strongly and usually showing mitoses in both myocardial and endocardial elements, and some invasion of the implant by host vessels was observed. The peculiar feature was that the host animal never survived the operation longer than 12 days, and that the host invariably died, i.e., its heart and circulation stopped, before the implanted heart ceased pulsating.

In the case of the eye experiments most of the implants were absorbed when placed in the pericardial cavity of the host, several survived but were much smaller than the eyes of the host at the end of 30 days when fixed for histologic study. When the implants were made in the head region, near the dorsal wall of the forebrain in trout embryos, several lived and developed until killed at the end of 80 days. In no case did we observe any evidence of any physicochemical antagonism between implant and host, and only minor histological changes occurred in certain tissues.

In the few liver and spleen experiments that survived the parasites or other causes, there appeared to be no physiological antagonism between the host and implant or any marked histological changes.

The incompatibility of the implanted heart and host, which resulted in the death of the host, seems to be due to the production of some chemical substance (waste or humor) by the implant which has an inhibitory effect upon the host, resulting in stoppage of the heart. This effect did not occur in the eye experiments or, as far as could be determined, in those of either the liver or the spleen, although in all the liver cases the actual amount of tissue implanted was much greater than in the heart experiments.

It seems from the results presented that an active implant, such as the heart, produces a specific chemical substance which inhibits the function of the same or different organs in the host, while less active organs either do not produce such a substance, or, if produced, it occurs in amounts too small to have any decided effect upon the host.

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PLATE I

FIG. 1.—120 hours after implantation, showing cell undergoing mitosis (*X*) in wall of ventricle. Oil immersion.

FIG. 2.—Regeneration of optic nerve (*ON*) and growth into wall of auricle (*A*). *L*, liver; *V*, ventricle. High power.

FIG. 3.—Growth of regenerated optic nerve (*ON*) into ectoderm (*Ec*). High power.

FIG. 4.—Growth of optic nerve (*ON*) through pericardium (*P*) into liver (*L*). High power.

FIG. 5.—Implant eye (*I*) normally developed in side of embryo posterior to normal eye (*E*). *Y*, yolk. High power.

FIG. 6.—Implant (*I*) in position between normal eyes. Low power.

PLATE II

FIG. 7.—Regeneration of optic nerve (*ON*) and the entrance of one branch (*1*), following blood capillary into brain (*B*). High power.

FIG. 8.—Drawing of foregoing section showing division of regenerated optic nerve (*ON*) into three branches (*1*, *2*, *3*). 39 days after implantation.

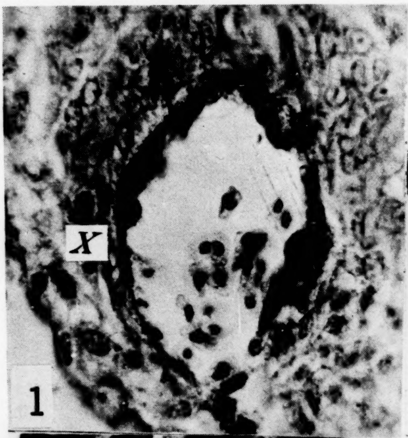
FIG. 9.—Drawing of the regenerated optic nerve (*ON*) passing around cartilage (*C*) which obstructed the nerve path. 104 days after implantation.

FIG. 10.—Implanted liver (*L*) fused with host liver (*HL*). Blood capillaries passed from host liver to implant. 72 hours after implantation. High power.

FIG. 11.—Implant liver (*L*) in yolk sac under mesoderm. Blood capillary (*BC*) sending branches (not shown) into implant. 72 hours after implantation.

FIG. 12.—Implant spleen (*S*) imbedded in mesoderm, adjacent to host liver (*L*). High power.

PLATE I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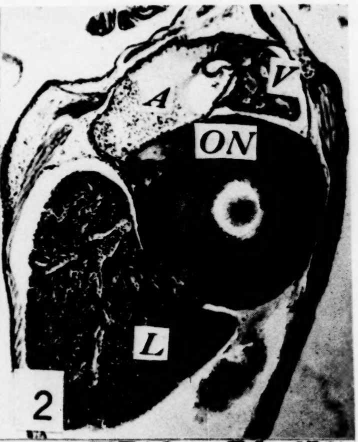
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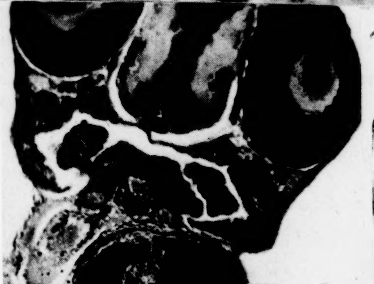
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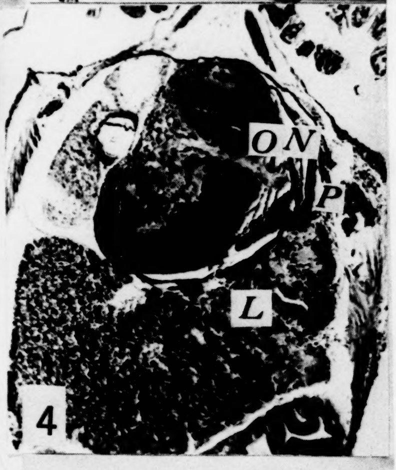
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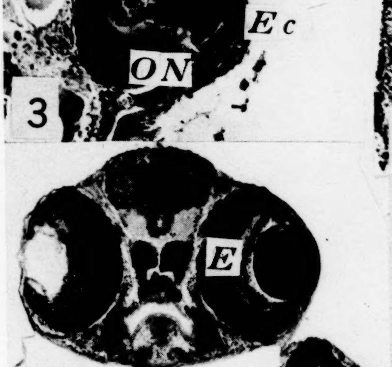
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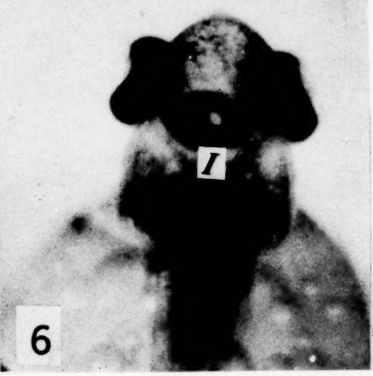
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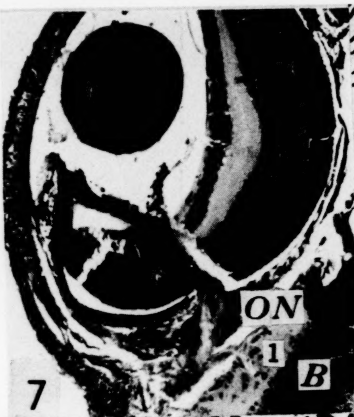
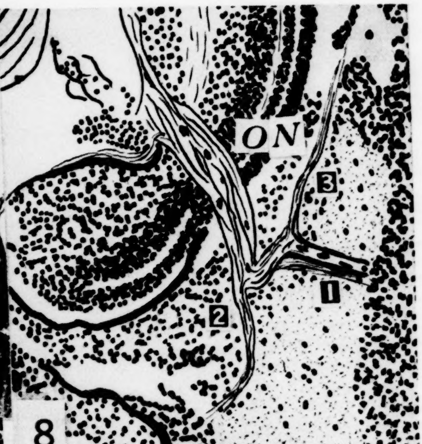
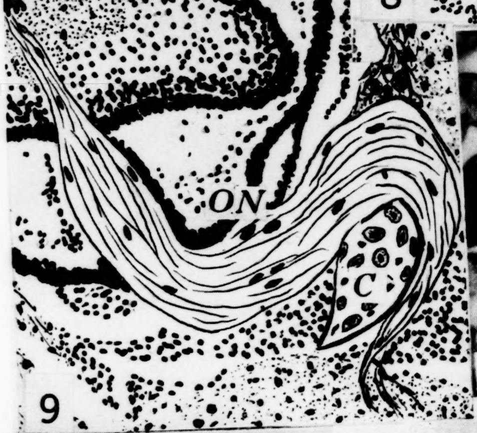
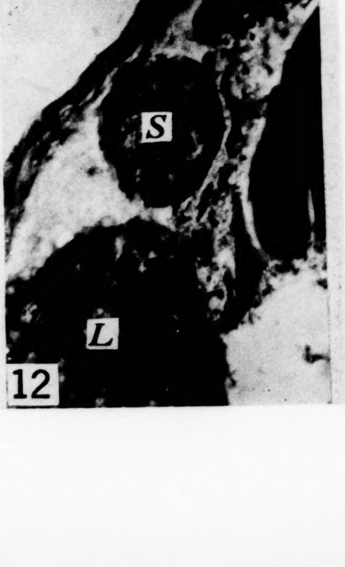
PLATE II









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CALCIUM AND POTASSIUM DEFICIENCY AS POSSIBLE CAUSES OF CERTAIN DELAYED FETAL MOVEMENTS

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ANGULO (1930, 1933*a*, 1933*b*, 1934) described spontaneous slow waving movements of the limbs of young rat fetuses occurring some time after ligating the umbilical cord. Presumably, as in other experiments (Angulo, 1932), the specimens were studied in a bath of physiological saline solution. He suggested that ligation of the umbilical vessels had caused "endogenous stimulation" of spinal motor centers by metabolic carbon dioxide, and proposed the theory that, "if the umbilical cord of any fetus is ligated, it will recapitulate the maximum degree of physiological activity it has achieved, but in the inverse order of its normal development."

While observing the same phenomenon in fetal rats, sheep, and cats, it seemed that the movements did not always manifest themselves in the orderly sequence indicated by Angulo's theory, and it appeared questionable that they were activated by the central nervous system. Certainly, they were not the first simple reflex-like movements which can be seen only under good physiological conditions before asphyxia begins (Windle, Orr, and Minear, 1934; Carmichael, 1934; Barcroft, Barron, and Windle, 1936). Therefore, it was decided to examine them in some detail to determine, if possible, their nature.

METHOD

Sixty-nine cat embryos and fetuses between 13.5 and 55 mm. C.R. length and 23–38 days' insemination age were obtained from animals previously decerebrated or under ether anesthesia. The umbilical cords were ligated or cut to allow bleeding; and the specimens were placed separately in small dishes of amniotic fluid (cat), distilled water, Locke's solution, 0.7 per cent sodium chloride, 0.9 per cent sodium chloride, or isotonic (6 per cent) sodium hexametaphosphate. Most of them were allowed to cool to room temperature, but a few were maintained at body temperature. They were watched constantly, usually by several observers, for periods of about 2–4 hours; and timed records of activities were kept.

RESULTS

The delayed spontaneous movements described by Angulo were observed in nearly all of the cat fetuses when the proper solutions were used. They were most commonly seen in the limbs and tail but, at one time or another, involved many other parts. Limbs moved at proximal or distal joints and the plane of movement varied; even the digits could be seen to react. Rotation of the head and flexion of the trunk were encountered less frequently; and movements of the ears, jaw, and abdominal muscles were observed in a few specimens. In the smallest, 13.5–14 mm. long, forelimbs were the only reacting parts. There was no consistent order of appearance of activities, even in fetuses of the same age and size, and thus no absolute relationship to the order of normal development of behavior. In some the tail, in others the wrist, elbow, shoulder, or hind-limb joints, were involved in the first movement.

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[1] Aided by a grant from the American Academy of Arts and Sciences.

Although isolated movements were often observed, they were usually repeated several or many times in rhythms of activity. Commonly, the tail would be flexed at the rate of once every 4 seconds, while a limb moved simultaneously in rhythm of one beat in 3 seconds.

The movements of the type under consideration were not encountered in fetuses whose placentas were still attached to the uterus with the circulation intact. As reported in a preliminary note (Windle and Orr, 1934), there was a relationship between time of delivering a fetus into the bath of physiological saline and the time of cutting the umbilical cord. The longer a fetus had been exposed to the salt solution before cutting its cord, the sooner the movements appeared afterward. When specimens were removed by severing their umbilical cords and placed in salt solution immediately after opening the uterus, movements began in about half an hour.

All fetuses placed in 0.7 per cent or 0.9 per cent sodium chloride, except one 13.5-mm. specimen, exhibited spontaneous activities. The movements continued long after the fetuses had cooled, their hearts had ceased to beat, and their reflexes could no longer be stimulated with strong faradic shocks. Spontaneous rhythmical movements were observed more than 5 hours after cutting the umbilical cord in one experiment. In distilled water a few strong reactions appeared within 20 minutes but soon stopped. Potassium-free Locke's solution gave results slightly less striking than did the sodium chloride solutions. In calcium-free Locke's, the responses appeared more slowly and were weaker; several fetuses showed no spontaneous movements in calcium-free Locke's solution. Those placed in the solution of sodium hexametaphosphate, which has the property of rendering calcium unusable physiologically (Danforth and Ivy, 1938), reacted very quickly; but movements did not persist long.

In cat's amniotic fluid the waving movements failed to appear. Similarly, Locke's solution brought about activity in no fetus smaller than 53 mm. C.R. length. Three of five 53–55-mm. specimens which had been placed in Locke's did execute weak tail and limb movements. However, three others from the same litter of eight placed in sodium chloride at the same time showed very strong responses of tail, limbs, head, and ears.

Fetuses of various sizes which had failed to move in Locke's solution or amniotic fluid were transferred to the other solutions. There they invariably became active. It took longer for movements to appear after transferring from amniotic fluid to sodium chloride than from Locke's to sodium chloride. Similarly, those which were active in calcium- or potassium-free solutions stopped moving when placed in Locke's. Transfers were repeated several times in a number of experiments, and the fetuses moved or became quiet according to the nature of the solution.

COMMENT

The spontaneous contractions of the developing skeletal muscle after ligating or cutting the umbilical cord were the result of alterations in the surrounding medium. They were not due to accumulation of metabolic carbon dioxide in asphyxia; had they been, they should have appeared as readily in amniotic fluid or Locke's as in other isotonic solutions.

The fact that they occurred in calcium-free Locke's and in sodium hexametaphosphate, which renders calcium inactive physiologically, suggests that a deficiency of calcium ions is one of the causative agents. The importance of proper calcium balance is well established in physiological investigations. That calcium unbalance causes spontaneous skeletal muscle-twitching has been observed by Jasper (1938). Lehmann (1937*a*, 1937*b*) demonstrated spontaneous discharge occurring when the threshold falls with an increase in alkalinity and when it is lowered by exclusion or deionization of calcium. Factors other than calcium deficiency probably play a part in bringing about these fetal movements. Potassium-lack seems to be one of them.

Whether the spontaneous rhythmical movements resulted from firing of neurons or muscle cells alone is open to some question. Faradic stimulation of the central nervous system was ineffectual, but muscles could be induced to contract by placing the electrodes directly over them; even so, the peripheral motor nerves may have been stimulated, for they are present early in development (Windle, 1937). If the occurrence of the movements in question were dependent upon ionic deficiency in the nerve cell itself, one would see no reaction until time sufficient for diffusion from the spinal cord had elapsed, and the great muscle masses of the trunk should be activated as soon and as often as the smaller, more distal muscles. This, very definitely, was not the case; distal movements were in the majority and were nearly always the first to appear. In fact, the very tip of the tail or the hand or foot were usually the sites of first activity. These were the thinnest parts, where diffusion could occur most easily. It is probable that peripheral motor mechanisms were involved.

The greater facility of ionic diffusion from thin parts of the embryo most recently developed may explain an apparent recapitulation of activity in the inverse order of normal development. However, in a series of sufficient length, instances will be found in which forelimbs move before the tail does, and head movements even may precede those of other parts.

CONCLUSIONS

1. Spontaneous contractions of skeletal muscles occurred in cat fetuses 13.5–55 mm. C.R. length placed in physiological saline solutions lacking calcium, potassium, or both. They occurred in isotonic sodium hexametaphosphate and in distilled water. They failed to appear in amniotic fluid and were rarely seen in Locke's solution.
2. Deficiency of calcium and potassium in the surrounding medium appears to be responsible for their appearance.
3. The reaction seems to take place in the peripheral mechanism rather than in the central nervous system. It may involve the skeletal muscles alone or the peripheral motor nerves or nerve endings.

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THE INTERACTIONS OF THE SLOW AND THE FAST CONTRACTION OF CRUSTACEAN MUSCLE

(Five figures)

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IN PREVIOUS publications (van Harreveld and Wiersma, 1936, and Wiersma and van Harreveld, 1938*a*) it has been shown that the adductor muscle of the chelipeds and walking legs of all decapod crustaceans so far investigated have a double motor innervation. They are innervated by only two motor axons, the stimulation of each of which causes a specific kind of contraction. The contraction caused by the stimulation of the thicker of the motor fibers is the fast contraction, and for this reason this nerve fiber will be called the "fast" fiber; and, correspondingly, the thinner fiber will be called the "slow" fiber, since it causes a slow contraction. Physiological and anatomical evidence shows that both the slow and the fast contraction can occur in the same muscle fiber (Wiersma and van Harreveld, 1938*b;* van Harreveld, 1938).

Facilitation — Facilitation

Nerve impulse → Transmission → Muscle action-current process → Transmission → Contractile substance

FIG. 1

A series of intermediary reactions can be distinguished in the transmission between the nerve impulse and the mechanical contraction (Marmont and Wiersma, 1938); a scheme for these various steps is given in Figure 1. Though all the steps can be demonstrated in both kinds of contraction, their relative magnitude differs. In marine crustaceans the action-current facilitation is pronounced in both kinds of contraction, whereas in *Cambarus clarkii* this facilitation is demonstrable only in special circumstances (Wiersma and van Harreveld, 1938*b*). In the same papers experiments have been described on the relation between the size af the muscle-action current and the mechanical contraction which made it clear that a second facilitation process exists between the action current and the mechanical contraction.

The process underlying the muscle-action current seems not to be propagated, nor all-or-none (Wiersma and van Harreveld, 1938*b*). The distribution of the excitation over the muscle fiber is not a function of the action-current process but is caused by the multiple innervation of the muscle fibers (van Harreveld, 1938). From anatomical evidence it is likely that the endings of the slow and the fast nerve fibers are intermingled on the surface of the muscle fiber.

These considerations make a mutual influence of the two kinds of contraction likely. In the following experiments the influence of fatigue of one kind of contraction on the other and the facilitation of one by the other were studied both with regard to the mechanogram and the action currents.

METHOD

The nerve fibers for the slow and for the fast contractions were prepared by splitting the nerve bundles, as has been described previously (van Harreveld and Wiersma, 1936). Each nerve fiber was stimulated by a separate pair of micromanipulated electrodes. The fiber eliciting the test contraction was usually stimulated at a low frequency, using a thyratron stimulating device which supplied rectangular currents of a short duration. The other fiber was stimulated faradically with an induction coil.

A. INFLUENCE OF THE FAST ON THE SLOW CONTRACTION

Cambarus clarkii and Astacus trowbridgii.—To investigate the influence of the fast on the slow contraction, the slow fiber was stimulated at a low frequency (10–20 per second) for periods of 3 seconds at regular intervals of 3 minutes. This rest of 3 minutes between two stimulations is enough to abolish the facilitation caused by the foregoing stimulation of the same fiber (autofacilitation). Accordingly, these contractions were very constant in size and speed; the only variation which ever occurred was a very gradual decline with the aging of the preparation. After a few contractions were obtained in this way, the stimulation of the slow fiber was preceded by a stimulation of the fast fiber. The best results were obtained by stimulating the fast fiber for 30 seconds (40–50 per second), with an interval of 15 seconds before the next stimulation of the slow fiber, to give the muscle time to relax from the fast contraction.

Two types of result were obtained. In a minority of cases (about 20 per cent) the faradization of the fast fiber caused at the first trial a marked depression of the following slow contraction. This depression was of a more or less permanent nature, for only a slight recovery was observed during the following sequence of test stimulations of the slow fiber. Repetition of the stimulation of the fast fiber resulted in a still more pronounced depression of the slow contraction, and in this way the slow contraction could be quite abolished for the frequency of stimulation used. On increasing the stimulation frequency, a slow contraction could be elicited again. We will discuss the nature of this depression later.

In the majority of the cases, instead of a depression there was a facilitation of the slow test contractions which followed the stimulation of the fast fiber (heterofacilitation). This facilitated contraction had a shorter latent period and a quicker rise than a nonfacilitated one and usually attained a greater height. When, instead of heterofacilitation, autofacilitation was produced by a stimulation of the same duration and frequency (40–50 per second), the test contraction was much more facilitated. This was even true when the frequency of the stimulus which caused the autofacilitation was the same as that used for the test contraction (10–20 per second). Figure 2 illustrates these phenomena. In a few preparations which initially showed a clear heterofacilitation, this diminished with repetition of the experiment and made way for the depression described above. In some others the facilitation was very poor at the first trial, and in the second trial, depression was obtained.

After complete exhaustion of the fast contraction, faradic stimulation of the slow fiber elicits a contraction which is not noticeable smaller than a similar contraction preceding the exhaustion of the fast contraction.

Marine crustaceans.—A large number of experiments has been performed on *Cancer anthonyi*, *Pagurus ochotensis*, and *Loxorhynchus grandis;* the results conformed with those obtained in *Cambarus* and *Astacus*. In all these species heterofacilitation was obtained; but autofacilitation was more pronounced, the reduction caused by a preceding stimulation of the fast fiber was occasionally found, and exhaustion of the fast contraction did not reduce the height of the following slow contraction. Small quantitative differences were found between the species investigated. For instance, in *Pagurus* the difference between auto- and heterofacilitation is relatively small because of strong heterofacilitation; the reduction by a preceding stimulation of the fast fiber was particularly pronounced in *Cancer*.

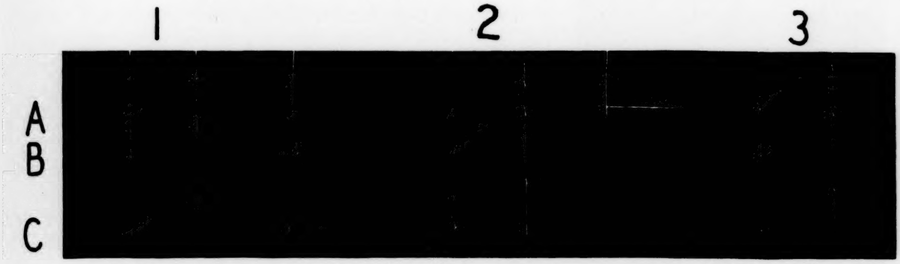

FIG. 2.—Heterofacilitation and autofacilitation of the slow contraction in *Cambarus*. The slow fiber is stimulated at a frequency of 15 per second for periods of 3 seconds with intervals of 3 minutes. In *A3* and *B2* the test stimulation is preceded by a faradic stimulation of the fast fiber for 30 seconds. Both heterofacilitated contractions are higher and steeper than the normal ones. In *C2* the test stimulation was preceded by a stimulation of the slow fiber for 30 seconds at a frequency of 15 per second; a distinct autofacilitation is visible.

B. INFLUENCE OF THE SLOW ON THE FAST CONTRACTION

Cambarus and Astacus.—After complete exhaustion of the slow contraction, faradic stimulation was applied to the fast fiber. The resulting contraction was not noticeably smaller than a similar contraction elicited in the fresh preparation; nor was it possible to suppress in this way the twitch contraction caused by a single induction shock applied to the fast fiber. Because it takes 10–15 minutes to exhaust the slow contraction, the reduction of the twitch which usually occurs during this period cannot be referred with certainty to an influence of the exhaustion of the slow contraction, since such a reduction can also take place by a decline of the twitch during this lapse of time without stimulation of the slow fiber.

To investigate whether the slow contraction has a facilitating influence on the fast, the latter had to be brought into a condition in which such a facilitation would be demonstrable. Under normal circumstances the twitch contraction is so strong that the small increase which might be expected from heterofacilitation would be hard to demonstrate. In previous communications (Wiersma, 1933; Wiersma and van Harreveld, 1938*b*) it has been stated that the twitch elicited by one single nerve impulse in the fast fiber diminishes gradually on repetition of the stimulus, ultimately disappearing completely (without an appreciable change in the action current). When the twitch contraction has disappeared, faradic stimulation of the fast fiber invariably elicits a large mechanical response, showing that autofacilitation is pronounced under these circumstances. This autofacilitation was further studied in the following experiment. The fast fiber is stimulated with shocks at a frequency of about 1 per second until the twitch has entirely disappeared. Then the

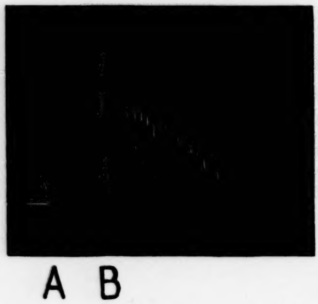

FIG. 3.—Autofacilitation of the fast contraction in *Cambarus*. At *A* the fast fiber was stimulated with 10 single induction shocks (1 per second); only on the first two is a visible contraction obtained. At *B* the frequency was suddenly increased to 40 per second for a period of 3 seconds. After 1 second the stimulation was resumed at a frequency of 1 per second; high twitches resulted, which gradually declined.

frequency is suddenly increased to about 50 per second for a duration of a few seconds, eliciting a tetanic contraction. Stimulation is then stopped for different periods and afterward resumed with a frequency of 1 per second. It was now found that such shocks will elicit contractions when started within a period of about 30 seconds after the high-frequency stimulation. This experiment is illustrated in Figure 3. The height to which the twitches are facilitated depends on a number of factors. If the duration of the high-frequency stimulation is too short, the effect is small; if it is too long, no effect at all is obtained, probably because of a preponderance of fatigue. In a certain preparation, for instance, only very small twitches were obtained 10 seconds after a high-frequency stimulation of 1 second; no contraction at all resulted after a similar 15-second stimulation period; but after a stimulation period of 5 seconds, large twitches occurred. In all cases the facilitating influence of the high-frequency stimulation becomes gradually smaller as the experiment is repeated.

To investigate the heterofacilitation the preparation was brought into a condition similar to the one described in the preceding experiment. After reducing the twitch contraction by repeated stimuli, short series (e.g., five) of stimuli at a frequency of one every 2 seconds were given with intervals of 1 minute. It was found that the height of the contractions thus elicited soon became fairly constant (usually with a gradual slow decrease with time). Each series consisted of a first, rather high contraction, followed by a much smaller, second one; whereas the later stimuli either elicited very small contractions or had no visible effect at all. After a fairly constant height of the different contractions was established, a period of 30 seconds of faradic stimulation of the slow fiber was given during the period of rest of the fast fiber, ending 15 seconds before the next series of stimuli was due. The effect of such a stimulation was, in almost every case, a very noticeable increase in the height of the following twitches (Fig. 4). Sometimes the facilitation was clearest in the first, some-

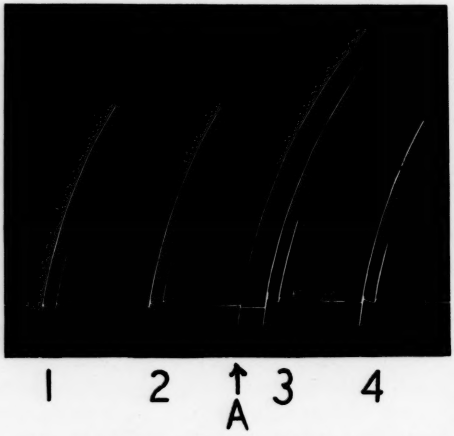

FIG. 4.—Heterofacilitation of the fast contraction in *Cambarus*. Four series of five stimuli applied to the fast fiber at intervals of 1 minute are shown. Only the first three stimuli of each series give a visible effect. The third series is preceded by a faradic stimulation of the slow fiber for 30 seconds. This contraction is recorded at *A* (drum stopped). All three visible contractions elicited by the next stimulation series are obviously facilitated. This heterofacilitation has almost completely disappeared in the fourth series of stimuli.

times in a later, member of the heterofacilitated row of contractions. Often a member of the stimulation series which did not elicit a visible contraction before the facilitation did so afterward. In a number of experiments the facilitation lasted longer than 1 minute, and sometimes could be detected for a period of 3 minutes.

Marine crustaceans.—Unlike *Cambarus*, *Cancer* and *Loxorhynchus* do not give a twitch contraction on a single nerve impulse in the fast fiber. The tetanic contractions, especially those with lower frequency of stimulation, develop much more gradually than in *Cambarus*, and it is thus possible to apply the same method as has been used to investigate the influence of the fast contraction on the slow. Usually stimulations of the fast fiber with a frequency of about 20 per second were given for a duration of 5 seconds with intervals of 3 minutes. Again the slow fiber was stimulated for a period of 30 seconds, ending 15 seconds before the next test stimulation of the fast fiber was due. Small but very distinct facilitations of the fast contraction by the preceding stimulation of the slow fiber were obtained in both species.

C. MUTUAL INFLUENCE OF THE MUSCLE-ACTION CURRENTS OF THE FAST AND THE SLOW CONTRACTION

A large number of experiments were performed in order to find an influence of the stimulation of one of the motor fibers on the muscle-action currents elicited by the stimulation of the other. *Cambarus*, *Astacus*, and *Cancer* were used in these experiments. The action currents were recorded under the same experimental conditions under which an influence of one kind of contraction on the other was found. Since *Cambarus* and *Astacus* do not usually show any autofacilitation of the action currents of the fast contraction (Wiersma and van Harreveld, 1938*a*), it could not be expected that they would show any heterofacilitation. In *Cancer* there is pronounced autofacilitation and a marked growth of the action currents during faradic stimulation of the fast fiber, and so heterofacilitation of the action currents might here be expected. However, the results of the experiments were quite negative: the action currents of the fast contraction started and grew in the same way, and to the same extent, before and after a facilitating stimulation of the slow fiber. Also, fatigue of the action currents of the slow contraction had no distinct influence on the action currents of the fast contraction.

In none of the three species was it found that the fast contraction had a significant effect on the action currents of the slow. In a few preparations a slight increase, in some others a slight decrease, of the action currents of the slow contraction following stimulation of the fast fiber seemed to be present. These effects, however, were so small that they cannot be considered as significant, since the preparations sometimes showed spontaneous variations of the same magnitude.

DISCUSSION

Since a distinct heterofacilitation was found for the mechanogram and not for the electrogram under the same circumstances, it is almost certain that the facilitation of the mechanical response obtained is not due to an increase of the action currents but to a facilitation of the transmission process between the action current and the contractile substance (see Fig. 1).

Though the effect of faradic stimulation of the fast fiber was usually a facilitation of the following slow contraction, in a number of cases a depressing influence was observed. In some preparations the facilitating and the depressing effects were even seen in the course of one experiment. It is likely that a fast contraction has, at the same time, a facilitating and a depressing effect on the slow contraction, and that the result obtained is the sum of these two influences. This is probably one of the reasons for the smallness of the heterofacilitation, compared with the autofacilitation, under similar conditions. Another reason may be that by autofacilitation the height of the action currents is increased (with the exception of those of the fast contraction in *Cambarus* and *Astacus*). Since the action-current process is a link in the chain of events connecting the nerve impulse with the mechanical response, an increase of the action currents will be usually accompanied by an increase of the mechanical contraction (Marmont and Wiersma, 1938).

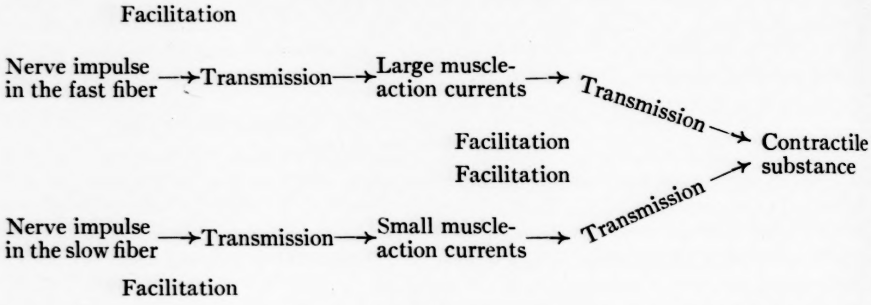
Facilitation
Nerve impulse in the fast fiber
Transmission
Large muscle-action currents
Transmission
Facilitation
Facilitation
Contractile substance
Transmission
Nerve impulse in the slow fiber
Transmission
Small muscle-action currents
Facilitation

FIG. 5

Since heterofacilitation of the action currents was not found, it must be concluded that the first part of the chain of intermediary processes of the slow and of the fast contraction, including the process underlying the action currents, are independent to such an extent that they even do not influence each other. This means that the intermediary processes of the fast and the slow contraction, including the action currents, are locally quite separated. From anatomical evidence it is likely that the numerous endings of the fast and those of the slow fiber do show local separation, which makes the separation of the beginning of the two chains of intermediary processes possible.

There are several arguments for the belief that the same contractile substance is involved in both kinds of contraction. It has been shown (van Harreveld and Wiersma, 1936) that in the adductor muscle of *Cambarus* the maximum tension which can be developed by each of the two kinds of contractions is approximately the same. It has been found, furthermore (unpublished), that the chemical changes in the muscle caused by the two kinds of contraction are qualitatively the same and about equal in magnitude when the two contractions are comparable in strength and duration. The fact that exhaustion of one of the contractions does not noticeably decrease the other seems opposed to this view. It has been shown, however, that during such stimulation the action currents diminish long before the contraction has reached its maximum, and that they become so small when the contraction starts to decline that this decline is most likely due to the fatigue of the action current (Wiersma and van Harreveld, 1938*b*). Therefore, it would not be possible to exhaust the contractile substance by faradic stimulation of the motor axons.

It has been mentioned that the twitch contraction in *Astacus* and *Cambarus* can disappear without a reduction of the action current or a fatigue of the contractile substance. It must be concluded that this depression is due to a change in the transmission process between the action current and the contractile substance. The depression, caused by a short faradic stimulation (30 per second) of the fast fiber, of a slow contraction elicited by low-frequency stimulation must be due to a similar block, since the action currents were not noticeably influenced by the preceding fast contraction.

The question whether the transmission processes between both action currents and the contractile substance are separated or common cannot be answered. Since these transmission processes can be mutually heterofacilitated, it seems certain that they are qualitatively the same, which is further supported by the fact that both transmission processes can be blocked by the same inhibitory fiber (Marmont and Wiersma, 1938; Wiersma and Marmont, 1938). A scheme of the intermediary processes involved in the double motor innervation is given in Figure 5.

SUMMARY

The mutual influence of the slow and the fast contractions of the adductor muscle of the chelipeds and walking legs of several fresh-water and marine crustaceans was studied.

Exhaustion of one kind of contraction did not decrease the action currents of the other; nor did one contraction produce a detectable facilitation of the action currents of the other.

Exhaustion of one kind of contraction did not noticeably reduce the mechanical response of the other. A distinct, but small facilitation of the mechanical response by a preceding faradic stimulation of the other motor fiber (heterofacilitation) was found.

It is concluded that the first parts of the chain of intermediary processes between the nerve impulse and the mechanical contraction, including the action currents, are locally separated in the two kinds of contraction, and that the heterofacilitation is located in the transmission process between the action current and the contraction. It is argued that the contractile substance is the same for both kinds of contraction.

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THE RESPIRATORY METABOLISM OF STARVED JAPANESE BEETLE LARVAE (POPILLIA JAPONICA NEWMAN) AT DIFFERENT RELATIVE HUMIDITIES[1]

(One figure)

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THIRD-INSTAR larvae of the Japanese beetle, *Popillia japonica*, were starved at different humidities by Ludwig and Landsman (1937), who found that the length of life varied directly with the humidity. At a low humidity of 0–5 per cent the larvae lived about 4 days, while at a high humidity of 96 per cent they lived about a month. It was also noted that their water content at death was very nearly constant (56–58 per cent), regardless of the humidity, indicating that the larvae died of desiccation. The only exception was found in the high humidity, where the larvae survived for a month and had a much higher water content (68 per cent) when they died. Hence, at this humidity death was due not to desiccation but probably to starvation.

Ludwig (1936) observed that Japanese beetle larvae lose water rapidly at the beginning of the desiccation period and that the rate of water loss progressively decreases. It was also noticed that a decrease in the activity of the larvae occurred and that they became quiescent after the first day. He suggested that the decrease in the rate of water loss after the first few days of desiccation may be associated with a decrease in the metabolic rate accompanying the quiescent condition. The relatively slow rates at which the prepupal and pupal stages become desiccated also support this view.

The effect of desiccation on metabolic rate differs in different insects. Caldwell (1925) found an increase in metabolism when the larvae of the mealworm, *Tenebrio molitor*, were desiccated to less than one-half of the possible limit. Continued desiccation caused it to fall far below normal. He suggested that desiccation had a stimulating effect on the metabolic rate. In the same paper Caldwell reported that under the same conditions the eight-spotted forester moth, *Alpia octomaculata*, did not show an increased metabolic rate. In this form it drops steadily to a low point when death occurs. Bodine (1933) subjected eggs of the grasshopper, *Melanoplus differentialis*, to hypertonic solutions and found a decrease in the oxygen consumption at all stages of desiccation. Ludwig (1937) observed a decrease in the metabolic rate when the nymphs of the grasshopper, *Chortophaga viridifasciata*, were starved at different relative humidities. In these experiments starvation caused death in 5 or 6 days, regardless of the humidity. Moreover, the degree of humidity exerts no significant influence on the rate of oxygen consumption, although at the high humidities water content increased to 82.4 per cent and at the low humidities it decreased to 58.8 per cent. Water content, therefore, had no influence on metabolic rate; but starvation caused a rapid decrease in metabolism, which became progressively lower until death. Since Japanese beetle larvae can withstand periods of starvation for as long as a month when maintained in an atmosphere of high humidity, it is very evident that their type of response must be different from that of *Chortophaga*. Hence, an experiment was devised to investigate changes in metabolism accompanying starvation of Japanese beetle larvae at different relative humidities.

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[1] The present experiments were undertaken at the suggestion of Dr. Daniel Ludwig, to whom the writer wishes to express his deep appreciation for the advice and generous assistance given him throughout the course of these investigations.

MATERIALS AND METHODS

Third-instar larvae of the Japanese beetle were collected in the field during the fall and early winter. Each individual was placed in a 1-ounce metal salve box containing moist soil of approximately the same humidity as the normal environment of the larvae. They were then stored in a refrigerator, where a low temperature of 8–10° C. prevented development and insured a ready supply of larvae during the winter months. Each larva was examined at intervals of several days. A few grains of wheat were added to serve as food, and sufficient water was added to maintain the proper humidity. Before subjecting the larvae to experimental conditions, they were removed from the refrigerator and kept at room temperature for a period of a week to acclimate them to the higher temperatures used in the experiment.

During the experiments the larvae, exclusive of fecal materials, were weighed each day on a chainomatic balance sensitive to 0.1 mg. Respiratory metabolism was measured by the modified Krogh manometer (Bodine and Orr, 1925). Readings were taken at a constant temperature of 25° C. Each larva was hung inside the manometer bulb in a tight-fitting wire basket to prevent excess movement. To determine oxygen consumption, 2 cc. of 2 per cent KOH were placed in the manometer bulbs to absorb the carbon dioxide. Readings were then taken over a period of several hours, after which the KOH was removed and the bulbs thoroughly washed. The manometers were then returned to the water bath, and the readings were again taken for several hours. From the difference between the first and the second sets of readings the carbon dioxide production and the respiratory quotients were determined. Readings of the respiratory metabolism were made on larvae exposed to relative humidities of 0–5, 56, and 96 per cent. These values were obtained by using the following substances in the bases of ordinary desiccators: for 0–5 per cent, anhydrous calcium chloride; for 56 per cent, a saturated solution of sodium bromide; and for 96 per cent, distilled water (Ludwig, 1937). The larvae used for the metabolism experiments were kept in the desiccators at the various humidities except for the daily period of 4 or 5 hours required for the respiratory readings. This daily exposure to the humidity of the manometer flasks probably did not materially change the water content of the tissues, since complete desiccation at the time of death showed approximately the same residual water as found by Ludwig and Landsman (1937) for individuals not used in the respiratory experiments. The desiccators were always kept at a constant temperature of 25° C. Only during the period required for weighing and adjustment in the manometers were the larvae exposed to laboratory temperatures. All experimental computations and conclusions in these experiments are based on data obtained from twenty or more larvae.

OBSERVATIONS

When the third-instar larvae of the Japanese beetle were starved at the humidities of 0–5, 56, and 96 per cent, they suffered a rapid loss in weight during the first day. At low humidities this rapid decrease continued until the larvae died; but at the higher humidities the rate decreased gradually, and finally there was a rather constant, small, daily weight loss. At humidities of 0–5 and 56 per cent the larvae died with approximately the same water content (53.2–54.5 per cent), while at the high humidity of 96 per cent individuals died when their water content had decreased to only 75.9 per cent.

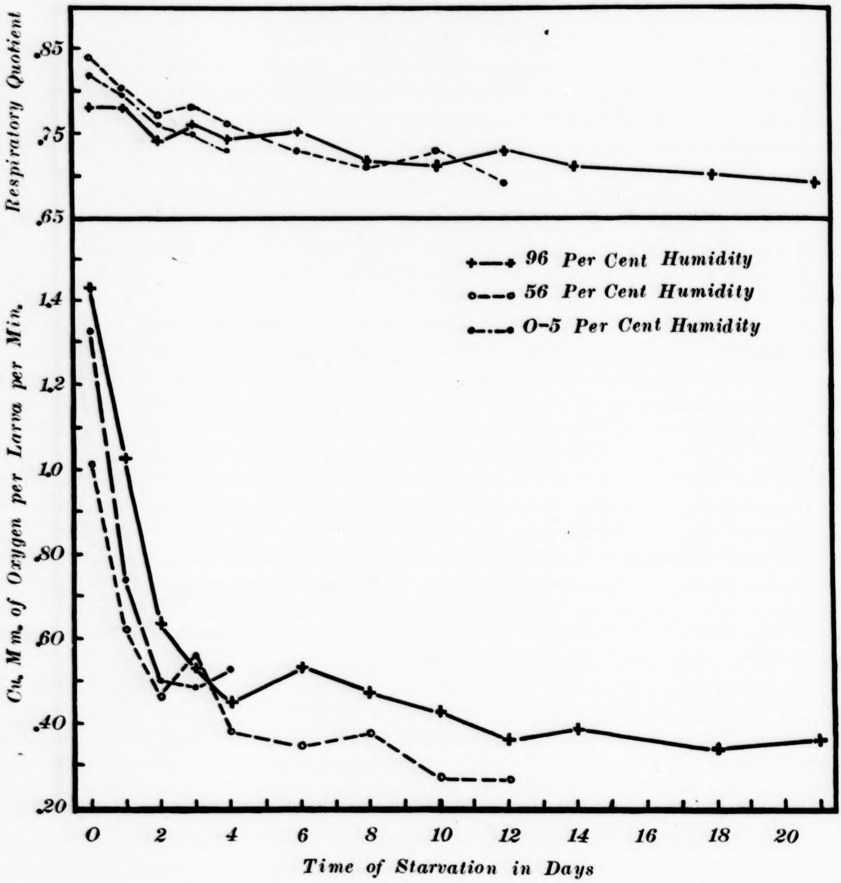

FIG. 1.—Changes in the metabolic rate of Japanese beetle larvae starved at different relative humidities.

Larvae at 0–5 and 56 per cent humidity died of desiccation in an average of 4 and 12 days, respectively; while at 96 per cent they died of starvation in an average of 26 days. The decrease in metabolic rate which accompanied starvation at each humidity is plotted in Figure 1. The amount of oxygen consumed by each larva decreased rapidly at the beginning of the starvation period. This rapid decrease, however, did not continue and after the second day metabolism remained at a low level, with very little change until death. A drop in the respiratory quotient was also observed. Starting at an average value of 0.82, a rapid decrease occurred for several days, and then the respiratory quotient fluctuated about 0.70 and remained at this value until death. Calculations were also made of the oxygen used per gram per minute. These results show the same trend as those shown in Figure 1, except at the humidity of 0–5 per cent, where an

TABLE 1

THE EFFECT OF HUMIDITY ON THE RESPIRATORY EXCHANGE OF STARVED JAPANESE BEETLE LARVAE

Hours of Starvation	Weight as Percentage of Original Weight	Water Content (Per Cent)*	O_2 Mm.^{3}/Min./Larva	O_2 per Larva as Percentage of Original	O_2 Mm.^{3}/Min./Gram	Percentage of Decrease in O_2 per Gram	R.Q.
	Calcium Chloride, 0–5 per Cent Humidity						
0	100.0	81.0	1.325±0.08	100.0	4.54	00.0	0.82
24	76.0	75.3	0.740±0.04	55.8	4.36	4.0	0.80
48	64.5	68.9	0.507±0.03	38.3	3.52	22.0	0.76
72	52.0	62.9	0.490±0.03	36.9	4.04	12.0	0.75
96	51.2	55.9	0.546±0.03	41.2	4.72	Above	0.73
	Sodium Bromide, 56 per Cent Humidity						
0	100.0	81.0	1.006±0.03	100.0	5.15	00.0	0.84
24	85.0	78.5	0.623±0.03	62.0	3.87	23.8	0.80
48	74.2	76.5	0.477±0.03	47.0	3.20	37.5	0.77
72	68.5	74.6	0.566±0.04	56.2	2.79	45.5	0.78
96	68.7	72.6	0.381±0.03	37.9	2.90	43.4	0.76
144	55.0	68.9	0.344±0.05	34.2	2.61	47.6	0.73
192	52.8	64.9	0.370±0.03	36.8	2.91	43.2	0.71
240	50.0	60.8	0.277±0.03	27.5	2.77	46.0	0.73
288			0.269±0.03	26.5	1.81	64.6	0.69
	Distilled Water 96 per Cent Humidity						
0	100.0	81.0	1.434±0.07	100.0	6.55	00.0	0.78
24	91.5		1.076±0.04	75.2	5.41	17.5	0.78
48	87.5	80.0	0.621±0.02	43.4	3.18	51.6	0.74
72	82.8		0.557±0.03	38.9	2.95	55.2	0.76
96	82.0		0.452±0.08	31.5	2.42	63.4	0.74
144	79.5		0.544±0.06	38.0	3.08	52.9	0.75
192	77.5	78.9	0.472±0.08	33.0	2.76	57.8	0.71
240	79.8		0.438±0.01	30.6	2.45	62.6	0.71
288	74.5		0.368±0.02	25.7	2.49	62.0	0.73
336			0.389±0.03	27.2	2.22	66.1	0.71
432		77.3	0.345±0.02	24.1	1.92	68.8	0.70
504			0.367±0.02	25.7	2.23	70.7	0.69
576			0.340±0.06	23.8	1.92	71.0	0.71

* The data in this column were obtained from unpublished work of Ludwig.

apparent increase in the metabolic rate is indicated after the second day. However, this apparent increase was caused by the very rapid loss in weight which accompanied starvation at the low humidity.

Table 1 contains a tabulation of the weight and water loss, the rates of oxygen consumption, the respiratory quotients, and the percentage of decrease in respiratory metabolism at different periods of starvation for each humidity. This table demonstrates a steady decrease in the metabolic rate regardless of the humidity or the water content of the larvae.

Table 2 shows that the metabolic rates after comparable hours of starvation at different humidities are, in most cases, not statistically different. However, it was observed that in several cases the difference between the means divided by the probable error of the difference between these means gives values of more than 3, indicating that the means are statistically different. Using the 240-hour stage of starvation in both the 56 and 96 per cent humidities, the value is 6.2. However, if reference is made to Table 1, it will be noticed that at 56 per cent humidity the metabolic rate was reduced 27.5 per cent of its original value, and that at 96 per cent humidity a reduction to 33.0 per cent occurred. This table also shows that the individuals kept at 56 per cent humidity used less oxygen per minute than those kept at 96 per cent humidity, and this difference was maintained throughout the experiment. In each case where the difference divided by the probable error of the difference gives values of more than 3, the means are statistically different, because the small oxygen consumption of small individuals (those used at the 56 per cent humidity) was compared with larger values observed for the larger individuals kept at 96 and 0–5 per cent humidities. Larvae approximately the same size never showed a statistical difference in the rate of oxygen consumption after comparable hours of starvation, regardless of the humidity. Therefore, water content of the larvae

TABLE 2

PROBABLE SIGNIFICANCE OF DIFFERENT RESPIRATORY READINGS OBTAINED AFTER VARIOUS PERIODS OF STARVATION AT DIFFERENT HUMIDITIES

Oxygen Consumption per Larva per Minute	Difference between Means	Probable Error of Difference	Difference/ Probable Error of Difference
48 hours at 0–5 and 56 per cent	0.030	0.049	0.6
48 hours at 0–5 and 96 per cent	0.114	0.040	2.8
48 hours at 56 and 96 per cent	0.144	0.037	3.9
72 hours at 0–5 and 56 per cent	0.076	0.055	1.4
72 hours at 0–5 and 96 per cent	0.067	0.043	1.6
72 hours at 56 and 96 per cent	0.009	0.053	0.2
96 hours at 0–5 and 56 per cent	0.165	0.044	3.7
96 hours at 0–5 and 96 per cent	0.094	0.083	1.1
96 hours at 56 and 96 per cent	0.071	0.083	0.8
144 hours at 56 and 96 per cent	0.200	0.024	0.8
192 hours at 56 and 96 per cent	0.102	0.092	1.1
240 hours at 56 and 96 per cent	0.161	0.260	6.2

seems to have no effect on the metabolic rate, and the decrease observed is caused by starvation.

DISCUSSION

Different species of insects manifest different susceptibilities to changes in humidity. Some can withstand very low humidities for a long time, while others need a very moist atmosphere for survival. Mellanby (1932*a*, 1932*b*) showed that the mealworm, *Tenebrio molitor*, can conserve metabolic water and thus withstand the very dry environment in which it lives. Conversely, Evans (1935) found that the prepupa and the pupa of the blowfly, *Lucilia sericata*, lose a constant amount of dry matter at all humidities, and that the greater loss of weight in dry atmospheres is entirely due to a loss of water. Similarly, Ludwig (1936) has shown that the larvae of the Japanese beetle are unable to conserve water derived from metabolic processes. Hence, in these experiments the weight loss was due almost entirely to a loss of water, except at 96 per cent humidity, where considerable amounts of tissue solids were utilized and where death was caused by starvation (Ludwig and Landsman, 1937). The rapid loss in weight that invariably occurred at the beginning of the experiments decreased gradually in the higher humidities, and finally there was a constant, small, daily weight loss; while at the lower humidity, death occurred before similar results could be observed.

Starvation at different relative humidities always causes a decrease in the respiratory metabolism. Bodine (1921) believed that starvation caused the nymphs of the grasshopper, *C. viridifasciata*, to lose water rapidly, dehydration causing death. However, Ludwig (1937) demonstrated that this form died of starvation just as soon at high as at low humidities, although at the higher value no lowering of the water content was observed. In the grasshopper, utilization of tissue solids was very rapid, and, regardless of the water content of the body, death occurred after an average of 5.2–6.6 days of starvation. From the beginning of the desiccation period the metabolic rates of the grasshopper nymphs decreased very rapidly, and continued to do so until death occurred. In the present experiments it was found that the metabolic rate of the Japanese beetle larvae decreased rapidly for only 2 days and then remained relatively constant at a low level until death.

In these experiments, as in the case of the grasshopper, it was found that the water content of the tissues did not affect the metabolic rate. Water content, however, influenced the time of survival. Because the metabolic rate dropped to a low value, it was possible for the larvae, at high humidities, to live as long as 26 days, finally dying of starvation. At humidities of 0–5 and 56 per cent desiccation caused death by lowering the water content of the body to approximately 54 per cent. The metabolic rates of the grasshoppers did not show this response, and, regardless of the humidity and water content, grasshoppers died of starvation. Ludwig and Landsman (1937) found the time of survival of the Japanese beetle larvae to be slightly longer for the same humidities used in these experiments. These differences may be explained by the greater amount of handling required in measuring the respiratory metabolism.

The natural differences in the type of development and in the feeding habits of the grasshopper and the beetle larva may be correlated with difference responses to starvation. The grasshopper requires constant feeding, and there is little storage of fats. Hence, death by starvation may occur in a short time. On the other hand, the Japanese beetle larva must store enough food for use during the pupal stage. Therefore, it contains large quantities of fats, which may be used under conditions of starvation. The fact that the respiratory quotient of these larvae fell from an average of 0.82 to 0.70 indicates the utilization of fat. The ability of the Japanese beetle larvae to survive starvation for nearly a month at high humidities is probably due to the rapid reduction of the metabolic rate to a low level, thus reducing the consumption of stored foods.

SUMMARY

1. The time of survival of the Japanese beetle larvae depends on the humidity. At 0–5 per cent they lived 4 days; at 56 per cent they lived 12 days; and at 96 per cent they lived 26 days.
2. In these experiments death was caused by starvation at a humidity of 96 per cent and by desiccation at the lower humidities. The larvae exposed to 0–5 per cent humidity died with an average water content of 53.2–54.5 per cent, indicating that death was caused by desiccation; and at 96 per cent humidity they died of starvation, with a water content of 75.9 per cent.
3. Starvation caused the metabolic rate to fall rapidly during the first 2 days and then more slowly, fluctuating about a low level, which was maintained until death. The rapid decrease in the metabolic rate to a low point, barely sustaining life, may be the factor allowing the larvae to live as long as a month at high humidities without food.
4. There is a decrease in the respiratory quotient from an average of 0.82 to 0.70, indicating the oxidation of stored fats during starvation.
5. Different relative humidities or the water content of the larvae have no direct effect on the respiratory metabolism. The observed fall in the rate of oxygen consumption is due to starvation.

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AXIAL SUSCEPTIBILITY TO SERUM ANTIBODIES IN EUPLANARIA

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IN *EUPLANARIA DOROTOCEPHALA* the fundamental polarity of the organism is in some way associated with an anteroposterior differential in susceptibility to cytolytic agents. The mass of evidence at hand permits no serious doubt of this fact. It is also a well-established fact that disintegration may be induced in *E. dorotocephala* by a wide variety of chemical and physical agents and conditions that appear to have nothing in common except the property of causing cytolysis (Child, 1928, 1930; Buchanan, 1930*a*, 1930*b*, 1935). It has further been shown that certain metabolic processes decrease in rapidity from anterior toward posterior within the zooids (Child, 1928) and that the ability of the organism to appropriate water varies in a similar fashion (Buchanan, 1930*a*, 1930*b*). These facts warrant the conclusion that cytolytic agents which reveal polarity do so by acting upon, or through, an anteroposterior quantitatively graded state of protoplasmic activity. From this line of evidence, then, polarity is associated with, or represented by, an anteroposterior differential in reactivity.

But the analysis of organic polarity still presents many problems. Among these is the question of the role played by protein metabolism. An attack on the problem of the properties of the proteins along the polar axis has been undertaken by one of us (C. A. L.). He proposed to inject into the rabbit antigenic material prepared, as described below, from living *E. dorotocephala*. It was assumed that the planarian proteins might be antigenic and that any antibody generated in the body of the rabbit would be specific for planarian proteins. With these assumptions it would follow that, if protein reactivity along the long axis of the planarian body is uniform or ungraded, cytolysis by such antibodies would occur simultaneously in all regions of the animal, or at least not follow a time progression from anterior toward posterior.

Accordingly, experiments were planned with these objectives. As the work progressed and it became apparent that, in the rabbit, antibodies destructive to planarian tissues may be so induced, the operations were extended to include attempts to identify the nature of the antibodies by testing their temperature stability and by other means, to determine the duration of the response of the rabbit to the planarian antigen, and the action of the antibodies on regeneration in planarian pieces. The present paper is confined to a report of the presence of antiplanarian antibodies in serum from treated rabbits, and the order of cytolytic disintegration in the planarian when subjected to serum from treated rabbits. The work of preparation of the antigens and the sera, and the handling of all experimental material, was performed by the junior author. The senior author co-operated in determining the effects of the sera, made certain other contributions, and prepared this report. In subsequent papers the junior author will report the further progress of the work.

MATERIALS AND METHODS

Healthy rabbits not previously employed for experimental purposes were selected. *Euplanaria dorotocephala* that had been starved from 6 to 15 days were first washed in several changes of tap water, followed by several washings in sterile 0.85 per cent NaCl. The planarians were then blotted between filter papers, ground in a mortar, and suspended in sterile 0.85 per cent NaCl, then extracted for 30 minutes at 4°–6° C. The suspension was centrifuged at 1,500–2,000 r.p.m. for 10 minutes, and the supernatant liquid used for the injections.

Ordinarily, 4 cc. was given at each injection, with three injections in each rabbit at 3-day intervals. The injections were given intravenously, intraperitoneally, or subcutaneously. Material to be injected intravenously was first filtered through a Berkefeld candle of *N* porosity; but it was found that, when so injected, it was highly toxic. In many cases death, apparently from protein shock, followed within 15 minutes. Accordingly, intraperitoneal or subcutaneous injection was usually employed.

The sera were prepared from blood drawn from the marginal vein of the ear from 4 to 42 days after the last injection of antigenic material. As stated above, only rabbits that had not been previously employed for experiments were injected. Serum prepared from normal healthy uninjected rabbits was used in control experiments.

The experiments reported here are divided into two groups. The first group was designed to test the viability of *E. dorotocephala* in the serum of treated rabbits, as compared with viability in the serum of control animals. In the second set of experiments the purpose was to trace the regional differences in susceptibility to the serum of treated rabbits. The general method employed in experiments of the first group was as follows: A series of identical dilutions of serum from normal and from treated rabbits was made up in tap water.[1] Lots of ten *E. dorotocephala*, carefully selected with respect to length, state of nutrition, and uniformity of previous care, were subjected in flasks to 50 or 100 cc. of each dilution of serum, both from injected and from normal rabbits. The range of dilutions is shown in Table 1. The solutions of sera were renewed every 24 hours. The time of appearance of the first sign of disintegration was noted for each lot of ten animals, and the course of the disintegrative process was observed at frequent intervals. The approximate time required for complete disintegration of all the planaria in each flask was also recorded. It was soon found that in the various dilutions of serum from normal rabbits no disintegration occurred at room temperature within the time required for complete disintegration of the planarians in the serum from treated rabbits. For this reason data on the controls are omitted from Table 1.

In the second set of experiments, designed to observe more closely the order of disintegration, watch glasses were prepared, each containing a single carefully selected planarian. In each watch glass 5 cc. of 1:40 serum from injected rabbit was poured over the animal, and sketches of its state of disintegration were made from time to time. The medium was changed every 24 hours. These, as well as the first group of experiments, were conducted at room temperature.

RESULTS

The effect of the various concentrations of serum from rabbits injected with the planarian antigen is shown in Table 1. Since no disintegration appeared in planarians treated with serum from normal rabbits under identical conditions of concentration and temperature within the time limits shown in the table, it is clear that the serum of treated rabbits contains an antibody which causes structural breakdown in living planaria. The data also show that the effectiveness of the antibody decreases with increasing dilution; in solutions containing 1 part of serum and 159 parts of tap water (omitted from the table) the cytolytic effect of the antibody is not apparent after 7 days of treatment. An inspection of the table also shows that the cytolytic action is more prompt in serum taken from rabbits recently injected with the antigen than in serum taken after a lapse of time.

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[1] Much work has shown that distilled water is itself a cytolytic agent (Buchanan, 1930*a*). In this laboratory, tap water appears to contain the appropriate concentrations of salts for normal maintenance of *E. dorotocephala*.

The contrast in behavior between animals in the normal serum and those in serum from injected rabbits is quite sharp. Almost immediately on application of the sera, those in the serum from treated rabbits twist about and show all signs of being irritated. Later they become less active than animals in normal serum; if inverted, they right themselves but do not move about. Much later they assume a curved position, with the dorsal surface convex. Shortly after this position is assumed, disintegration begins. No such behavior is noted among animals subjected to the same concentrations of the normal serum. Although somewhat less active than in tap water, they otherwise behave quite normally.

TABLE 1

DISINTEGRATION OF *Euplanaria* IN VARIOUS CONCENTRATIONS OF SERUM FROM RABBITS INJECTED WITH *Euplanaria* EXTRACT

Expt. No.	Planaria		Serum Drawn Days After Last Injection	1–10		1–20		1–40		1–80	
	Size (Mm.)	Starved (Days)		Disintegration Began (Hr.)	Disintegration Complete (Hr.)	Disintegration Began (Hr.)	Disintegration Complete (Hr.)	Disintegration Began (Hr.)	Disintegration Complete (Hr.)	Disintegration Began (Hr.)	Disintegration Complete (Hr.)
I	12–18	14	17	......	......	60	72	60	84	70	98
II–1	19–20	5	6 and 8	24	52	37	52	44	84	84	68% intact in 168
II–4	12–15	7	14	23	49	23	61	36	97	None in 97	....
II–9	14–17	27	42	20	48	24	94	94	148	None in 148	....
III–2	12–15	12	10	44	80	48	114	80	150	None in 148	....
IV–1	12–15	7	4	41	72	48	96	68	140	96	30% intact in 170

The order of disintegration was observed in the watch-glass experiments with the aid of a hand lens or a binocular microscope. In all, eighty specimens were studied. In sixty-five cases disintegration began at the extreme anterior end. After the erosion of about one-fourth of the animal, proceeding from anterior toward posterior, disintegration began at the extreme posterior end. Complete disintegration was reached when that proceeding from anterior toward posterior met with the disintegration proceeding anteriorly, invariably in the region just posterior to the mouth. Of the remaining fifteen, several exceptionally long animals began to disintegrate at the posterior end before anterior disintegration appeared. In others, also long animals, some disintegration occurred in the vicinity of the mouth before anterior disintegration began. The general picture of the disintegrative process in these animals treated with serum from injected rabbits was very similar to that observed during disintegration by potassium cyanide, distilled water, absence of oxygen, and other cytolytic agents as described by other observers.

It has been shown that certain cytolytic agents are ineffective in the presence of hypertonic solutions (Child, 1930; Buchanan, 1930*b*). In the present work the osmotic pressure of the various concentrations of sera was not determined. But there is no reason to believe that the differences observed between the effects of normal and experimental sera may be due to differences in osmotic pressure. The osmotic pressure of rabbit blood is given as 6.809 atmospheres. The highest concentration employed here was 1 part serum and 9 parts water, and comparisons were made between identical concentrations of normal and experimental sera. No doubt such solutions are distinctly hypertonic, but they are not sufficiently so to prevent the water uptake associated with cytolysis.

DISCUSSION

We have to consider, first, the fact of cytolysis by the antibodies in the serum from treated rabbits, and second, the fact that this cytolysis begins almost invariably at the anterior end of the planarian and proceeds posteriorly before the posterior end begins to disintegrate. Thus, so far as the time factor is concerned, all regions of the animal are not equally susceptible to the cytolytic agent.

Evidence, to be presented in some detail in later papers, indicates that the antisubstance in the serum of treated rabbits is protein; at least it is as heat-sensitive as are the proteins. But data associating it with a specific fraction are not yet available. In the absence of evidence to the contrary we may follow the conventional interpretation of the nature of the action of antigenic materials and assume that planarian proteins, or certain of them, induce in the rabbit the production of precipitins,[2] probably protein in character. In the present state of the work it is not possible to state whether one or several precipitins occur in the serum from treated rabbits. Until analyses now in progress are more complete, it may be assumed that the observed cytolysis is a consequence of the precipitation of planarian proteins by the antibodies.

There is also insufficient knowledge at hand as to whether the action of the antibodies on planarian proteins is due to a specific chemical interchange or to the adsorption of the one by the other. In either event it is quite clear that those substances, presumably proteins, which are acted upon by the antibodies are more reactive at the anterior end of the animal than elsewhere and that the reactivity decreases uniformly from each end toward the region immediately posterior to the mouth, where reactivity is least and cytolysis occurs last.

In specimens of *E. dorotocephala* as long as those employed here, the body consists of more than one zooid (Child, 1909). The posterior limit of the most anterior zooid is approximately that region last to undergo cytolysis. When the order of disintegration by the antisubstance is considered in the light of this fact, it becomes clear that the anterior zooid is characterized by a gradient in reactivity of the proteins, or certain of them.

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[2] Heating to 55°–56° did not destroy the planariacidal properties of the serum from treated rabbits, but precipitation tests with planaria extracts have not yet been completed.

The imposing mass of data concerning the graded differential in protoplasmic activity associated with the fundamental polarity of *E. dorotocephala* has been reviewed elsewhere (Child, 1928, 1930; Buchanan, 1930*a*, 1930*b*, 1935). It includes evidence of gradients in susceptibility to lipoid solvents, to high temperature, to anesthetics and other depressants, to lack of oxygen, to calcium precipitants, to hydrion concentration, in ability to appropriate water, in carbon dioxide production and oxygen utilization, and other evidences of protoplasmic activity. All lines of evidence agree in indicating that the anteroposterior axis of the zooids is characterized by an anteroposterior decrease in rate of metabolic processes. The present contribution adds the fact that the reactivity of the proteins also decreases from anterior toward posterior. Several interpretations of this fact are possible. It is difficult to assume that the antibodies attack an anteroposterior series of different proteins, each progressively less reactive than the next anterior one, for this involves assuming that the serum from treated rabbits contains an almost infinitely great number of antibodies or that a single antibody is nonspecific. On the other hand, assuming the antisubstance to be highly specific, as is usually the case with immunity bodies, we have to account for the fact that it attacks the proteins of the anterior region first. Either protoplasmic structure is increasingly less dependent upon proteins anteroposteriorly or the proteins are increasingly more protected from precipitins from anterior toward posterior. Work now under way is planned to determine the nature of the proteins which show this differential.

The question as to which, if any, of the many metabolic processes which show this differential in rate along the polar axis is the master reaction upon which organic polarity depends remains unanswered. The line of approach indicated here holds out promise of important contributions to a more complete understanding of the nature of the metabolic gradient and, through it, of the nature of organic polarity.

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THE EFFECTS OF SUPERSONIC VIBRATIONS ON RECONSTITUTION AND HEAD FREQUENCY IN EUPLANARIA DOROTOCEPHALA[1]

(Five figures)

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MANY experiments have been performed dealing with susceptibility, reconstitution, and the frequency of the regeneration of heads in relation to a large number of physical and chemical agents using the fresh-water flatworms as experimental animals. As regards the words "regeneration" and "reconstitution," the use of both is justified. The head and posterior end *regenerate*, but the piece as a whole undergoes *reconstitution* into a whole animal. Regeneration is one feature of reconstitution. Properly used, the two words do not mean the same thing, though many use the word "regeneration" for all reconstitution. Such agents as dilute KCN (Child, 1916), anesthetics (Buchanan, 1922), caffeine (Hinrichs, 1924), mechanical stimulation (Child, 1920), and changes in temperature conditions influence reconstitution.

Two factors are concerned in head regeneration in *Euplanaria dorotocephala* (Child and Watanabe, 1935): the physiological condition of the cells directly concerned, and the physiological condition of other regions of the piece posterior to the region of the developing head. Other works (Child, 1916; Behre, 1918; Buchanan, 1922; and Hinrichs, 1924) also have shown that inhibiting agents acting on the head-forming cells tend to decrease head frequency. The action of such agents on other parts of pieces cut from *Euplanaria* tend to increase head frequency. The results depend on the relation between these two factors and the period of exposure to the agent.

By "head frequency" we mean the frequency of the various head forms that have reconstituted from lots consisting of pieces of a certain length and from a certain body-level of animals of the same length. If a given lot of pieces shows a majority of normal or near-normal animals, the lot is characterized as having a high head frequency. A lot giving a majority of headless or strongly inhibited head forms is characterized as having a low head frequency.

Wiercinski and Child (1936) obtained evidence which indicated that supersonic vibrations, primarily a mechanical factor, produced the disintegration gradients such as had been observed with other agents. These results suggested the possibility that supersonics might affect head frequency.

MATERIALS AND METHODS

Euplanaria dorotocephala, found in freshwater springs near Chicago, was used as the experimental animal. The worms were kept in the laboratory in large granite pans filled with well water, were fed liver twice a week, and the water was changed five times a week, care being taken to remove all liver debris.

Before experimentation several pans of worms were kept without food for 2 weeks, since this procedure has been found to give greater uniformity in experiment. Lots of 60 animals each, of uniform size, were selected from the starved stock for the control and experimental groups. For head-frequency studies the animals were cut in transverse sections, as shown in Figure 1. Only the anterior zooid was used, and the pieces were approximatey one-eighth the length of the animal, exclusive of the head. Of the 60 pieces cut from each region, those that showed the greatest variation in length were discarded. In the majority of cases, 50 pieces were used. This procedure decreased any error that might be attributed to the difference in length of the pieces. The test and control animals were sectioned in well water. The test pieces were placed on the quartz crystal with a wide-mouth pipette, either immediately after section or after periods of delay, were exposed to supersonic vibrations, and were returned to well water immediately after exposure. Child (1916) described five classes of regenerated pieces, depending on the degree of inhibition, and these have been commonly used in such studies since that time; hence, the definition of each class can be omitted here. The classes and the arbitrary values assigned to each type of head were: normal, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; and acephalic, 20. To obtain the "head-frequency index," these arbitrary values were multiplied by the frequency of each type of heads; and the sum of all was divided by the total number of living animals. The "mean," described by Child and Watanabe (1935), is identical with the foregoing head-frequency index.

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[1] The author wishes to thank Professor C. M. Child for advice and criticism received throughout the progress of this work.

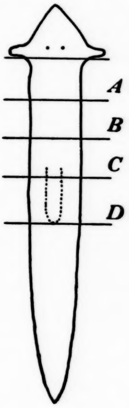

FIG. 1.—Levels *A*, *B*, *C*, and *D*, used in all head-frequency experiments.

The apparatus employed is a Hartley oscillator. The appendix in a paper by Harvey, Harvey, and Loomis (1928) gives the essentials for constructing the oscillator. The supersonic vibrations were produced by activating an octagonal piezoelectric quartz crystal 5.0 mm. in thickness and 4.5 mm. in width and length, giving a natural frequency of 612 kilocycles per second. The oscillating circuit is tuned to approximately the frequency of the crystal.

The crystal was mounted between two brass electrodes cut and milled from a $\frac{3}{8}$-inch brass plate of the same length and width as the crystal. This was set on the stage of a grounded microscope. For observation of supersonic phenomena a lower electrode was used, which had a circular opening to permit reflected light to come through. For exposing pieces of worms a solid electrode was used. The upper electrode is cut in the shape of a frame having a $\frac{3}{8}$-inch border with a lead to the grounded point. When set down upon the crystal, the upper electrode forms a chamber or electrode water cell.

The lower surface of the upper electrode was smeared with a thin film of vaseline, and the chamber was filled with water, in which the material is exposed. Intensities and exposure periods were determined by preliminary experiments. A medium intensity, as indicated by a reading of 5 amperes in the primary circuit and 100 milliamperes in the secondary circuit, was found most satisfactory.

The water temperature in the electrode cell rose approximately 6° C. during the average exposure required, but this rise was decreased to approximately 2° C. by the introduction of a glass capillary-tube cooling system. Control experiments with a similar rise in temperature showed no appreciable effect during the $2\frac{1}{2}$–4-minute exposures.

THE EFFECT OF SUPERSONIC VIBRATIONS ON INTACT ANIMALS

Groups of 75 and 5 worms were used in each test for determining the resistance of a large group, as compared with few individuals. The four relative intensities which were used in the scale from 4 to 7 amperes (reading in the primary inductance circuit of the oscillator) showed the lower resistance of few individuals, as contrasted with the higher resistance of many individuals composing a group. These results are graphically shown in Figure 2. The median relative intensities were employed in a scale of 0–10, to gain some indication as to the trend of intensity effect upon the groups.

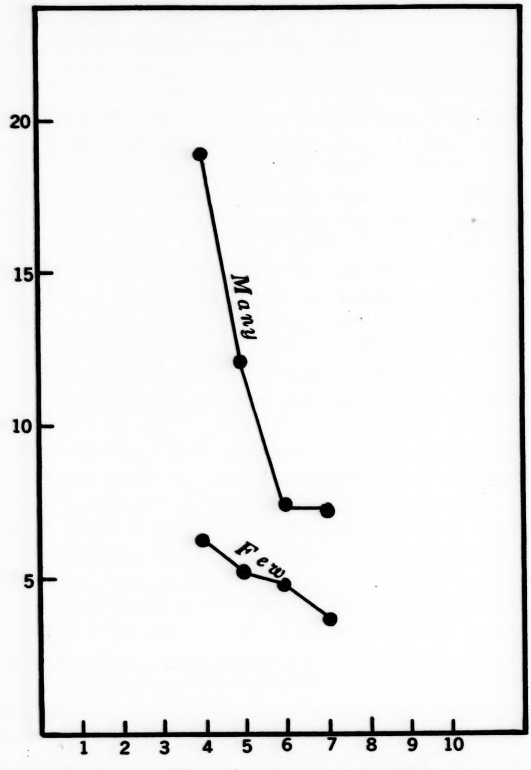

FIG. 2.—Comparison of resistance of few (5) and many (75) individuals in a group exposed to supersonic vibrations.

The course of disintegration in the individual worms when only a few were exposed showed the characteristic pattern of differential susceptibility. With prolonged exposure the pharynx is everted through the dorsal body wall.

In the lots of 75 worms, cytolysis was not uniform. In some individuals both head and posterior zooid region disintegrated, the pharyngeal region remaining intact; complete reconstitution occurred after a few days. In others, head and prepharyngeal regions disintegrated, pharynx and posterior zooid regions were intact, and a new head regenerated. In still others, the posterior zooid regions disintegrated, the anterior zooid remained intact, and reconstitution occurred.

The average for the different experiments for each intensity showed that for a few individuals undergoing supersonic treatment there is only a slight rise in disintegration time as the intensity is decreased. With many individuals the average shows a decided protection to the group as a whole, and it is well above the level of the plotted curve for few individuals. With a decreased intensity the massed individuals show a curve that rises rapidly, which means a great increase in disintegration time.

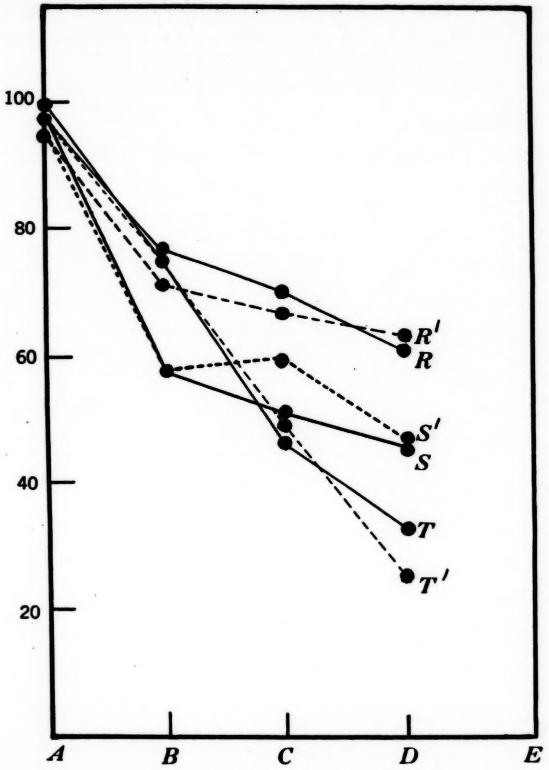

FIG. 3.—Head frequencies of pieces of worms from three different lots exposed to supersonic vibrations immediately after cutting. *R*, control; *R'*, experimental (14–16 mm.). *S*, control; *S'*, experimental (10–12 mm.). *T*, control; *T'*, experimental (12–14 mm.).

The lots of grouped individuals tend to come together in masses, and dense secretions of mucus are formed. This is caused by the physical contact of worms and the rapid vibration of the water. Such aggregation affords protection to the individual worms in the group. This adds another instance of group protection to the large number of such cases which have been reported by Allee and his associates (Allee, 1931, 1934). One may conclude that the following experiments with pieces in groups are affected as aggregations rather than as isolated pieces.

EFFECT OF EXPOSURE IMMEDIATELY AFTER SECTION

Experiments were first performed to determine whether supersonic treatment would affect the reconstitution of pieces cut from worms. The fourteen experiments involving 1,000 worms, exposed at an intensity of 5 amperes in the primary circuit and 100 milliamperes in the secondary circuit. The animals were collected at various times during the year. The supersonic treatment lasted from 2½ to 4 minutes. The duration of the exposure varied for the different lots, depending upon the time required to cause cytolysis of ten *A* pieces. The exposure time that would not cause visible cytolysis was applied to pieces *A*, *B*, *C*, and *D* (approximately successive fourths of the anterior zooid).

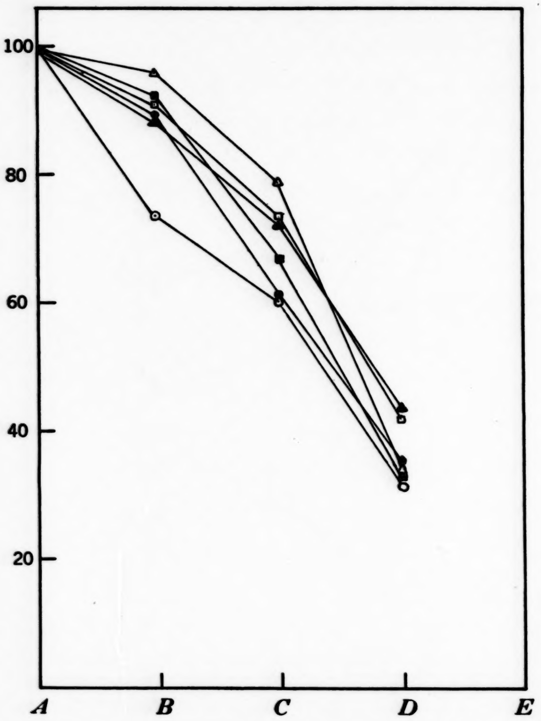

FIG. 4.—Head frequencies of 10–12-mm. worms with delayed exposure. Open circle, control; solid circle, 10 hours' delay before exposure; solid square, 18 hours' delay before exposure; solid triangle, 24 hours' delay before exposure; open square, 30 hours' delay before exposure; and open triangle, 42 hours' delay before exposure.

Although the head frequencies of the pieces of the different stocks did not always coincide (Rulon, 1936), the tests are about the same as the controls, except that in the 10–12-mm. stock of *C* pieces there is an increase (Fig. 3). Experiments in which the death-rate is high often raise the question whether a change in head frequency which may appear is real or due to the death of those which would have a lower head frequency if they had lived. There is an increase in the above-mentioned *C* pieces, and it seems to be real, since there are no deaths in the control and only 2.4 per cent deaths in the experimental lot. The index of the control is 50.9; that of the experimental lot, 59.9. In the 14–16-mm. pieces the slightly lower head frequency in pieces *A*, *B*, and *C* is perhaps an inhibition of the head-forming cells but is not sufficient to be significant.

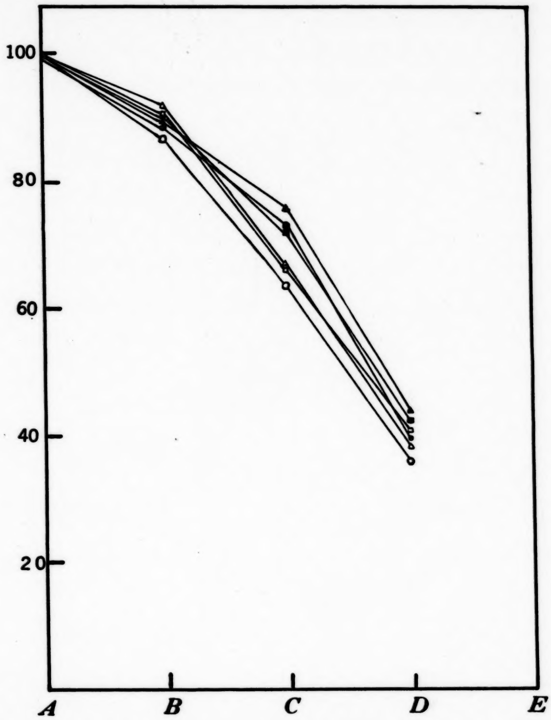

FIG. 5.—Head frequencies of 12–14-mm. worms with delayed exposure. Open circle, control; solid circle, 10 hours' delay before exposure; solid square, 18 hours' delay before exposure; solid triangle, 24 hours' delay before exposure; open square, 30 hours' delay before exposure; and open triangle, 42 hours' delay before exposure.

DELAYED EXPOSURE

Twenty-four experiments, including 1,900 worms, were performed with various periods of delay between section and exposure. Each experiment consisted of a control and test following 10, 18, 24, 30, and 42 hours' delay. The duration of exposure was determined as stated above. Head-frequency indexes and percentages living are tabulated in Tables 1 and 2, and Figures 4 and 5 show the increases for the two sizes of worms used in the experiments in *B*, *C*, and *D* pieces. The increase becomes greater as the period of delay increases, except that with the longest periods the index decreases in some cases.

The pieces taken from 10–12-mm. worms showed the greatest increase in *B* and *C* pieces with a 42-hour delay, and in *D* pieces with 24-hour delay (Table 2); those from 12–14-mm. worms in *C* and *D* pieces with 24-hour delay, in *B* pieces with 42 hours' delay. The tables show that in a few of the lots the death-rate is high, and these are therefore open to suspicion. However, the increase occurs in the lots with few or no deaths as well as in those with high death-rates.

TABLE 1

DELAYED EXPOSURE: 800 WORMS, 10–12 MM.

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Control	99.4	73.4	60.0	31.1	92.3	99.1	98.1	94.3
10-hour delayed	97.9	89.1	60.2	34.8	47.5	75.0	89.5	86.7
18-hour delayed	100.0	91.9	66.3	31.4	27.1	87.5	70.9	78.6
24-hour delayed	99.1	88.6	71.9	43.4	89.1	97.1	68.3	80.6
30-hour delayed	99.5	90.3	73.0	41.7	75.5	89.0	92.0	72.5
42-hour delayed	99.3	95.3	78.6	34.4	83.8	93.0	73.5	72.1

TABLE 2

DELAYED EXPOSURE: 1,100 WORMS, 12–14 MM.

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Control	99.8	86.8	63.6	36.0	100.0	100.0	89.2	95.0
10-hour delayed	97.5	88.1	72.4	39.3	37.3	75.3	92.9	81.8
18-hour delayed	99.4	89.5	72.3	42.0	91.5	98.6	97.4	100.0
24-hour delayed	98.8	88.6	75.4	43.5	75.2	89.7	91.6	89.7
30-hour delayed	100.0	89.7	66.4	40.6	85.3	86.2	96.6	97.5
42-hour delayed	100.0	91.1	66.7	37.7	97.5	97.9	81.4	81.6

CONCLUSION

The increase in effect on head frequency with delayed exposure probably means that, after exposure immediately following section, recovery occurs so rapidly that head frequency is only slightly or not at all affected. The apparent decrease of the head-frequency index with the longer period of delay may mean that, as regeneration of the head progresses, the cells giving rise to the head become more susceptible. The inhibiting effect on the physiological state of these cells begins to appear in head development.

The factor which inhibits head development in short posterior pieces is apparently a stimulation resulting from the posterior cut, and at least in considerable part it is nervous in character (Watanabe, 1935). The observed increase in head frequency presumably results from an inhibition or decrease of this stimulation, but how supersonic vibrations determine this effect is not known. As regards head frequency, supersonic waves act essentially like other inhibiting agents. Their effect on pieces with low head frequency is manifested to a greater degree on the factor inhibiting head development than on the head-forming cells. Further experiments may more definitely indicate the manner in which the effects of supersonics on organisms are produced.

SUMMARY

1. With a certain range of intensity and duration of exposure, supersonics bring about cytolysis in whole individuals of *E. dorotocephala*. This cytolysis progresses from the anterior end posteriorly in the anterior zooid, just as is the case when the worms are exposed to certain other physical and chemical agents.
2. On return to water after partial cytolysis, reconstitution occurs.
3. Worms exposed in groups of few individuals are more susceptible than in groups of many individuals.
4. In general the effect increases with intensity and duration of exposure, but individual differences occur as with other agents.
5. For determination of the effect on head frequency, experimental and control lots of pieces *A*, *B*, *C*, and *D* (approximately successive fourths of the anterior zooid) were used, since these pieces give progressive decrease and a wide range of head frequency from *A* to *D*. Effective intensities and exposure times were determined by preliminary experiment. Lots were exposed immediately after section and following various periods of delay.
6. With exposure immediately after section little change in head frequency occurred.
7. With delayed exposure an increase in head frequency occurs and is apparently greater with certain periods of delay than with longer or shorter periods.

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TESTS FOR SEXUAL DIFFERENTIATION IN PARAMECIUM MULTIMICRONUCLEATUM AND PARAMECIUM CAUDATUM

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CONJUGATION of a number of races of *Paramecium multimicronucleatum* Powers and Mitchell has been found to be correlated with environmental changes (Giese, 1935 and 1938). Sonneborn (1937) has found that for induction of conjugation in certain races of *P. aurelia* Müller two strains, which he calls "sexes," are necessary.

This paper summarizes attempts to determine (1) if sexual differentiation is present in any or all of the strains of *P. multimicronucleatum* and *P. caudatum* Ehrenberg in use in this laboratory and (2), if so, what effect, if any, environmental changes play when such sexual differentiation is present.

EXPERIMENTAL

Races 2E, 2C, 6, 7, 8, 9, 11, and 13 (most of these previously described [Giese, 1938]) of *P. multimicronucleatum* and races 4, 12A, 12B, and 14 of *P. caudatum* were used in this study. Stock cultures were maintained in the manner previously described (Giese and Taylor, 1935). Isolation cultures were grown in small tubes, 7 cm. long and 4 mm. in inner diameter, in the same medium and with the same precautions as for the larger cultures. Temporary slides were made with acetocarmine; permanent mounts were made with Schaudinn's fixative and the Feulgen reaction.

In a race showing genotypic sexual differentiation, progeny of a single individual should not conjugate without the reorganization of nuclear apparatus such as occurs during endomixis. If the progeny of a single individual can conjugate among themselves, either sexual differentiation in the rigid sense is absent or sex reversal among some of the members of a given genotype must be induced before conjugation can occur. Single animals of a race to be tested for sex were isolated into small tubes; and, after a number of divisions had occurred, one vigorous culture was chosen as stock. From this stock one individual was inoculated into each of a set of five to fifteen tubes. This was repeated from five to fourteen times for each of the various races tested. Stranghöner (1932) found that complete reorganization after endomixis may take as long as ten generations in *P. multimicronucleatum*. To determine whether endomixis was occurring in our cultures, samples were stained daily in some cases, at the time of transfer (about every 48 hours in most cases) in others. Examinations for conjugation were made every 12 hours; and in case no conjugants were observed in a given culture, the examinations were continued for 6 days after subculture, at which time the cultures were abandoned, as the animals were minute and not likely to conjugate.

Growth occurred rapidly in the small tubes, and the data on division-rate for three races of *P. multimicronucleatum* and for two races of *P. caudatum* are given in Table 1. In Table 2 are summarized data on tests for sex.

Examination of Table 2 shows that progeny of a single individual conjugate without intervention of endomixis in all of the races of *P. multimicronucleatum* except 2C but

TABLE 1

DIVISION-RATES OF A NUMBER OF RACES OF *Paramecium*

Species	Race Number	Time (in Hours) after Inoculation	Trials	Average Divisions per Day
Paramecium multimicronucleatum	2C	24	171	2.5
		48	91	2.3
		72	79	2.0
		96	32	1.6
	6	24	92	2.9
		48	47	2.8
		72	39	2.4
	9	24	79	2.6
		48	25	2.5
		72	47	2.4
P. caudatum	12A	24	48	3.1
		48	23	2.6
		72	20	2.4
	12B	24	50	3.1
		48	25	2.7
		72	26	2.5

TABLE 2

CONJUGATION AMONG PROGENY OF A SINGLE VEGETATIVE INDIVIDUAL DIVIDING WITHOUT INTERVENTION OF ENDOMIXIS

Species	Race Number	Number of Series Studied	Total Number of Trials	Percentage of Total Trials Showing Conjugation
Paramecium multimicronucleatum	2C	5	60	0
	2C	7	21	0
	2E	5	25	100
	6	14	266	(50)*
	7	12	85	100
	8	5	46	74
	9	6	51	100
	9	12	228	(75)*
	11	5	66	87
	13	5	25	25†
P. caudatum	4	5	25	0
	14	7	28	0
	12A	7	35	0
	12B	7	35	0

* In percentage of the series; data not kept for each trial. Both of these series were started from a single exconjugant.

† Cultures of race 13 were never very healthy and multiplication was always slow.

not in any of the races of *P. caudatum*. When conjugation occurred, it began, at the earliest, 72 hours after subculture. Individuals of races 8, 11, and 13 divided more slowly than the others, and in such cases conjugation was correspondingly delayed.

Progeny of a single exconjugant of race 6 were used in the experiments reported here on this race. After a period of very rapid multiplication, the division-rate dropped quickly, until finally, even after transfer to fresh medium, there were fewer than 0.5 divisions per day and experimentation was abandoned. Fourteen series were studied in all; in the first five, no conjugation occurred; thereafter conjugation occurred in seven of the nine remaining series. Stained preparations, either temporary or permanent, were made daily. No endomixis was observed to occur. However, macronuclear breakdown of the type described as "hemixis A" by Diller (1936), unaccompanied by micronuclear activity and macronuclear reorganization, was observed to occur. This macronuclear breakdown was found only among some of the offspring of a given individual. Similar but less marked macronuclear fragmentation was observed in occasional members of races 2 and 11 but not in any of the other races studied.

One of the cultures developed from isolated individuals of race 2 and its subcultures consistently showed conjugants without intervention of endomixis; others did not. The conjugating clone (labeled 2E) and one of the nonconjugating clones (labeled 2C) were isolated for further study. When in a series of six experiments 50 members of each race were mixed together and kept at about 20° C., 84 per cent of the animals in the mixed cultures conjugated; whereas, in controls (100 individuals) of 2E alone, 64 per cent of the animals conjugated; and in controls of race 2C alone, no conjugants were observed to occur. Whereas they would not conjugate among themselves, individuals of race 2C were conjugating with individuals of race 2E. When a single individual of 2E was placed in a culture of 2C, one and only one pair of conjugants was obtained. When 5 individuals of 2E were placed in a culture of 2C, never more than 5 conjugants were obtained (for five experiments: 3, 4, 5, 5, 5). Individuals of 2C cannot conjugate with one another but will conjugate with individuals of 2E; to this extent we may speak of sexual differentiation in this race.

If 2E and 2C are to be considered as opposite sexes, one might expect one or the other of them to mate with individuals of the other races of the same species. In a number of trials mixtures were made of individuals of races 7, 8, 9, and 11 with 2C, and also with 2E, at the time when each of the races tested was in a state of incipient conjugation (sticky state). Individuals of race 2 (E or C) can be distinguished from the individuals of the other races by the fact that the excretion crystals are much larger and usually much more numerous than in the other races. In no case were conjugants obtained in which a member of race 2 was conjugating with a member of any of the other races. This either indicates incompatibility or inadequate control of environment, although an attempt was made to mix only animals in incipient conjugation. Conjugants occurred in the mixtures, but examination showed members of pairs to be of the same race. Conjugants were obtained also in the unmixed controls except in cultures of race 2C.

Individuals of race 2E show a peculiar type of sexual "segregation" following vegetative divisions. For, if one isolates a pair of individuals in incipient conjugation, then separates them, and grows a culture from each individual, mixtures of the two cultures (50 individuals of each race were mixed; controls consisted of 100 of the unmixed individuals of each race) show a higher percentage of conjugation than does each culture separately under the same environmental conditions. Data for the first series are summarized in Table 3. Similar tests (four trials) were made upon cultures obtained by isolating each of the individuals of two pairs in incipient conjugation in race 2Eg (Table 3) of the first series. Another series of tests (five trials) were made upon two similar pairs from one of the races of the second series (race 2Eg1) and a final set of similar trials with one of the pairs of incipient conjugants obtained from 2Eg1. The data from these series are omitted for want of space, but they are comparable to the data in Table 3 for the first series. As indicated in the data in Table 3 for mixtures of members of different pairs and for similar mixtures in other series, members of the different pairs are like or unlike one another. The data as a whole suggest correlation of "sex" not with the macronucleus but with something less regularly distributed in vegetative division, possibly the micronucleus, since Stranghöner (1932) found such irregular distribution of the micronuclei in division.

TABLE 3

PERCENTAGE IN CONJUGATION IN MIXED AND UNMIXED CULTURES DERIVED (WITHOUT INTERVENING ENDOMIXIS) FROM EACH OF THE INDIVIDUALS OF INCIPIENT PAIRS OF CONJUGANTS OF RACE 2E OF *Paramecium multimicronucleatum*

TRIAL	PAIRS									MIXTURE OF MEMBERS OF DIFFERENT PAIRS			
	1			2			3*	4*	5*				
	a‡	b	a×b	c	d	c×d	e	f	g	af	ag	bg	ef
1.....	0	0	70	28	0	78	0	0	0	......	0	36	58
2.....	0	26	50	20	4	84	4	0	4	......	0	78	92
3.....	0	0	82	0	26	96	0	0	8	......	0	68	26
4.....	0	0	80	0	50	22	0	0	0	60	0	70	0
5.....	0	4	58	0	10	6	0	0	2	18	0	66	2
6.....	0	0	64	0	18	70	10	16	8	64	4	56	76
7.....	0	8	94	2	14	92	20	0	16	82	0	80	84
8.....	24	4	50	2	4	60	20	6	2	10	66†	78	20

* The other member of each of these pairs failed to give rise to a healthy culture.

† Reason for sudden change not known.

‡ The letters under each of the numbers refer to the members of a pair.

In none of the series of trials with the four races of *P. caudatum* was conjugation ever observed to occur in cultures descended from a single individual without the intervention of endomixis.[1] Tests and observations were made in the same manner as for the other species. However, when two of the races, labeled 12A and 12B, were mixed at the right state in development or grown together, conjugants were obtained in large numbers. A large number of tests with 15-cc. cultures indicated that conjugation was never obtained unless the two strains were present. This is like the situation described by Sonneborn (1937) for some races of *P. aurelia*.

Individuals of race 4, however, when mixed with either of the strains of race 12, did not conjugate. Similar tests with race 14 showed no conjugants, as did also mixtures of

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[1] After endomixis in race 12B sexual differentiation occurred and conjugants were obtained.

4 and 14. To make sure that the proper period for conjugation was not missed, experiments were tried at various times after subculture; and, also, animals of each of these races were grown together: 4 with 12A, 4 with 12B, 4 with 14, 14 with 12A, and 14 with 12B. The incompatibility of the sexes of race 12 with races 4 and 14 is at present incomprehensible. It is possible that some peculiar environmental condition necessary for conjugation was not supplied.

Where there is no clear-cut differentiation into sexes, as in races 6, 7, 8, 9, 11, and 13, the onset of conjugation is correlated with a definite environmental change (Giese, 1935, 1938), conjugation occurring only when there is a sudden decline in the nutritive conditions after a period of plenty. It is of great interest to determine if for the races showing sex differentiation (2E and 2C of *P. multimicronucleatum*, and 12A and 12B of *P. caudatum*) this is true, or whether, as in one of the races of *P. aurelia* described by Sonneborn (1937), individuals full of food will conjugate. To this end the following experiments were performed.

Individuals of race 2E, when still full of food, were added to similar individuals of race 2C. No immediate reaction occurred, and it was only after starvation that conjugants were obtained. Conjugants occurred for a period of 3 days; when pairs appeared, they were removed. It was demonstrated that 84 per cent of the animals had conjugated; but the epidemic was prolonged, the animals pairing only when they had reached a certain physiological state.

If both sets of paramecia had been starved subsequent to feeding, the clumping was immediate upon mixture, and within 2 hours the majority had fused in pairs; a count after 5 hours indicated that 88 per cent had paired. Such experiments were repeated many times with the same general result.

Results indicating similarly the importance of the environment were obtained in another series of experiments in which animals of 2E were introduced into cultures of 2C, starved to a point where they would quickly unite with properly starved members of race 2E. If 1 individual of 2E is introduced, a single clump of sometimes 10 or more members of 2C form about this animal. When the successful animal of 2C begins to pair with 1 individual of 2E, the others cease clumping about the pair. If one introduces 5 animals of 2E into a culture of 2C, generally 5 clumps are formed, never more than 5. If conjugants of 2E × 2C or 2E × 2E are introduced, there is no clumping. If dividing individuals of 2E are introduced, there is no clumping. If an individual of 2E, in the proper physiological state for conjugation but somewhat injured (large vacuole appearing within), is introduced, clumping occurs; but if the individual is killed by heat or by ultra-violet light, it no longer attracts the individuals of 2C. If an individual of 2E in the proper state for conjugation is placed into a suspension of the food bacterium and then, after the formation of food vacuoles, is quickly rinsed and placed with individuals of 2C, clumping occurs, followed by the usual pairing-up. Apparently, while the well-fed individual must be starved to achieve a certain state before it can conjugate, a mere stuffing with bacteria does not change the physiological state conducive to conjugation. A certain period must elapse after the food is taken in to change the physiological state. If too long a time elapses after feeding, the "attractive state" is lost, for a well-starved animal of 2E no longer causes clumping in a culture of 2C. The clumping observed in the experiments suggests some diffusible attractive substance; but, so far, attempts to isolate any substance in agar or in other ways have failed.

The results clearly indicate that, when sex is present, and at least members of race 2C must be mixed with 2E to obtain conjugation, both races (sexes) must be in the proper physiological state and in the proper environment before they will unite in conjugation.

Very similar results were obtained with *P. caudatum.* When well-fed individuals of strains 12A and 12B were mixed, no conjugants were observed until depletion of food was evident. When the animals were in the proper physiological state for conjugation, i.e., starved after a period of plenty, clumping occurred immediately after mixing, and pairing-up followed within 2–5 hours. If well-fed individuals of 12A were mixed with properly starved individuals of 12B, conjugants were not obtained; and the reciprocal experiment gave similar results. If either strain was starved too long, even if the other were in the proper physiological state for conjugation (as determined by mixing with the opposite sex in the proper physiological state and obtaining conjugants), conjugation failed to occur; and if both strains were starved too long, similar failure to conjugate was observed.

Environmental changes alone fail to induce conjugation when only one sex is present (cf. studies on race 2 reported in Giese, 1938), but environment may condition the physiological state conducive to conjugation even when sexual differentiation is present. It is also possible that certain environmental conditions may favor or suppress sex reversal in a given genotype. A knowledge of the effect of variations in the environment upon conjugation is therefore important for an understanding of the nature of the factors inducing conjugation.

SUMMARY

1. The majority of the races of *P. multimicronucleatum* tested showed conjugants even without the intervention of endomixis. In one race distinct separation into "sexes" was found.

2. Of four races of *P. caudatum,* two were found to be of opposite "sexes," conjugating only on mixture; the other two were not observed to conjugate with each other or, when mixed, with either of the other two strains.

3. In races in which conjugation occurred among progeny of a single individual without intervention of endomixis, the pairing followed exhaustion of food after a period of plenty.

4. Correlation of onset of conjugation and the exhaustion of food was also observed in the races showing separation into "sexes," for mere mixture when the animals were well nourished did not result in conjugation until the food supply was relatively depleted.

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THE QUESTION OF AUTOTROPHIC NUTRITION IN EUGLENA GRACILIS

(Three figures)

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THE trophic nature of *Euglena gracilis* has been investigated from time to time over a period of nearly forty years and is still a disputed question. Zumstein (1899) and Ternetz (1912) concluded that inorganic media are unfavorable to growth of *E. gracilis*. Pringsheim (1912), on the other hand, reported that this species grew almost as well in certain inorganic solutions as in media containing organic food, and Mainx (1928) likewise concluded that the species is facultatively autotrophic. Dusi (1933*a*), moreover, carried the Mainx strain through several successive transfers in inorganic media. More recently, Hutner (1936) has been unable to grow *E. gracilis* in such media, whereas Dusi (1937) has reaffirmed his earlier statements.

The need for a chlorophyll-bearing species which could be used as an autotrophic control in certain investigations on nutrition of colorless Euglenida led the writers to attempt the growth of *E. gracilis* in inorganic media. The failure of our first few attempts afforded no explanation for the contradictory results of Dusi and Hutner and has necessitated a more extensive investigation with several different inorganic media.

A bacteria-free strain of *E. gracilis*, obtained in 1931 from Professor E. G. Pringsheim, has since been maintained in pure culture in our laboratory. This strain was the source of the flagellates used in the present investigation. Satisfactory media for stock cultures have been described elsewhere (Hall, 1937*a*). The media used in the experiments on autotrophic nutrition are listed on page 77. When necessary, each medium was adjusted so that the pH after sterilization would fall within the optimal range (pH 6.5–7.5) for *E. gracilis* (Jahn, 1931; Dusi, 1933*a*). The solution was then tubed in measured amounts and sterilized in the autoclave at 15 pounds pressure for 20 minutes.

In the first transfer of each experimental series the culture tubes were inoculated from a stock flask or tube containing the appropriate inorganic medium and previously seeded from a stock culture of *E. gracilis*. In two series, specified below, the stock flask was inoculated from an agar slant culture by means of a platinum loop, so that very little organic material was carried over from the original culture. In other series the stock flask was inoculated by means of a graduated pipette from a culture in peptone medium. Each tube of the first transfer received a 0.5-cc. or, in certain cases, a 1.0-cc. inoculum from the stock flask. In each transfer approximately fifteen tubes were inoculated. Four of these were fixed for the initial count (number of flagellates per cubic centimeter), one was used for determination of the initial pH, and the remainder were incubated at room temperature near a window. After incubation four tubes were fixed for the final count, one was used for determination of final pH, and another for inoculation of the second transfer. The third transfer, in turn, was inoculated from a tube of the second transfer; and the same procedure was followed in subsequent transfers.

All pH determinations were made with a La Motte roulette colorimeter. Initial and final counts were made with a Whipple ocular micrometer and a Sedgwick-Rafter counting cell, as described previously (Hall, Johnson, and Loefer, 1935). In the calculation of peptone concentrations (grams per cubic centimeter) in successive transfers, the utilization of peptone by the flagellates was not considered. Hence, the concentrations listed below are conservative estimates of the peptone present.

Medium D ("solution D" of Mast and Pace, 1933):

NH_4Cl	0.497 gm.
$MgSO_4$ (anhydrous)	0.048 gm.
K_2HPO_4	0.209 gm.
Sodium acetate	1.148 gm.
Distilled water	1.0 liter

Medium EA (used by Loefer, 1934, for *Chlorogonium*):

NH_4NO_3	0.5 gm.
KH_2PO_4	0.5 gm.
$MgSO_4 \cdot 7H_2O$	0.1 gm.
NaCl	0.1 gm.
$FeCl_3$	0.0025 gm.
Distilled water	1.0 liter

Medium EAB: same formula as medium EA but contains, in addition, $MnCl_2 \cdot 4H_2O$ in a concentration of 5×10^{-7} molar.

Medium EC (Mainx, 1928; Dusi, 1933*b*):

$(NH_4)_2HPO_4$	1.0 gm.
K_2HPO_4	0.2 gm.
$MgSO_4 \cdot 7H_2O$	0.1 gm.
$FeCl_3$	0.0025 gm.
Distilled water	1.0 liter

Medium EF:

NH_4NO_3	1.0 gm.
$MgSO_4 \cdot 7H_2O$	0.2 gm.
$CaCl_2$	0.1 gm.
KH_2PO_4	0.2 gm.
$FeCl_3$	0.0025 gm.
Distilled water	1.0 liter

In addition, this medium contained $MnCl_2 \cdot 4H_2O$ in a concentration of 5×10^{-7} molar.

GROWTH OF *Englena gracilis* IN INORGANIC MEDIA

Series I and II (*medium D*).—In these preliminary series a stock flask seeded from an agar slant culture was used for inoculating tubes of the first transfer. The peptone concentration in the culture tubes was thus reduced to 2.1×10^{-7} in series I and 3.6×10^{-7} in series II. The initial pH was 7.0 in each case, and the initial counts were 683 per cubic centimeter in series I and 340 in series II. After 17 and 19 days of incubation, respectively, x/x_0 (ratio of final to initial count) was 18.4 in series I and 15.0 in series II, indicating moderate growth in each case. The second transfers were incubated for 17 days, when no growth was noted in either series. Since the calculated peptone concentrations were 1.3×10^{-8} in series I and 2.5×10^{-8} in series II, it was decided to avoid such an abrupt change from organic to inorganic media in subsequent experiments.

Series III (*medium D*).—Initial pH was 6.5 in the first transfer and 6.9 in the second and third. Initial counts were: 4,893, 3,542, and 280, respectively. Incubation periods were 14 days in the first transfer and 21 days in the second and third. Calculated peptone concentrations were 5×10^{-5}, 2.5×10^{-6}, and 1.2×10^{-7} in successive transfers. The results (Table 1) showed moderate growth in the first transfer and none in the second and third. The series was discontinued after the third transfer.

Series IV (*medium D*).—The initial pH was 6.8, 6.9, and 6.8 in successive transfers; and initial counts were: 14,733, 1,087, and 147. Calculated peptone dilutions were the same as in series III. Incubation periods were 13, 21, and 21 days. In the first transfer (Table 1) growth was comparable to that in the first transfer of series III; in the second, the apparent increase in number was not statistically significant; and in the third transfer there was a definite decrease in number of the flagellates. The results obtained in series I–IV thus indicate that medium D will not support growth of *E. gracilis* through successive transfers.

Series V (*medium EA*).—The initial pH was 6.6, 6.8, 6.9, and 6.8 in successive transfers; and initial counts were: 1,653, 8,075, 587, and 106. Calculated peptone dilutions were 5.2×10^{-5}, 3.3×10^{-6}, 1.6×10^{-7}, and $8 \times 10^{9-}$. Incubation periods were 8, 12, 9, and 22 days, respectively. Good growth (Table 1) occurred in the first transfer, poor growth in the second, and a decrease in number of flagellates was evident in both the third and fourth.

TABLE 1

SERIES III–IX: GROWTH OF *Euglena gracilis* IN INORGANIC MEDIA

Growth in the successive transfers of each series is expressed as x/x_0 (ratio of final to initial concentration of flagellates per cubic centimeter).

SERIES	x/x_0 IN SUCCESSIVE TRANSFERS, DIFFERENT SERIES			
	First	Second	Third	Fourth
III	6.5	1.0	0.8	....
IV	6.8	1.3	0.5	....
V	45.7	2.6	0.4	0.3
VI	41.4	2.8	1.5	62.4
VII	18.6	1.9	8.7	....
VIII	2.3	1.1	1.2	0.6
IX	13.5	1.0	13.7	1.0

Series VI (*medium EC*).—The initial pH was 7.0, 7.0, 6.9, and 7.0 in successive transfers; and the intial counts were: 680, 3,190, 620, and 53. Calculated peptone concentrations were: 4.5×10^{-5}, 2.2×10^{-6}, 1.1×10^{-7}, and 5.5×10^{-9}, respectively. Incubation periods were 8, 12, 21, and 18 days. The results (Table 1) showed good growth in the first and fourth transfers and slight increase in number of flagellates in the second and third transfers. Unfortunately, the departure of the junior author made it necessary for us to discontinue this series in the fourth transfer. The encouraging results led us to try the same medium in later experiments, one of which proved successful.

Series VII.—In this case ammonium phosphate was omitted from medium EC. Calculated peptone concentrations were approximately the same as for the first three transfers in series VI. Initial counts were 551, 633, and 93. Initial pH was 7.0, 6.9, and 7.0; and the incubation periods were 11, 12, and 21 days, respectively. Growth was apparent in each transfer, although much less in the second than in the other two. The results indicated that growth in the first three transfers may be attributed to the peptone carried over from the original stock culture.

Series VIII (*medium EC*).—Initial pH was 7.0, 7.1, 6.9, and 7.1 in successive transfers; and initial counts were 2,245, 3,900, 153, and 360. Calculated peptone concentrations ranged from 5×10^{-5} in the first transfer to 6×10^{-9} in the fourth. In inoculation of the fourth transfer, concentrated material from one tube of the third transfer was inoculated into another tube, and the latter was used for inoculation of the fourth transfer. In this way the initial count was increased to 360. The transfers were incubated for 17, 18, 20, and 21 days, respectively. The results (Table 1) showed poor growth in the first transfer, none in the second and third, and a decrease in number of flagellates in the fourth. The conflicting results of series VI and VIII were puzzling until it was discovered that different preparations of ammonium phosphate had been used in the two series. In a later series (XII), medium EC was made up with the same type of salt as that used in series VI, and the results showed better agreement with those of the latter series.

Series IX (*medium EAB*).—The addition of manganese to an inorganic medium was suggested by earlier observations (Hall, 1937*b*) that growth of *E. anabaena* is accelerated by this substance in certain concentrations. In this series initial pH was 7.1, 6.9, 6.9, and 6.7 in successive transfers; and initial counts were: 2,245, 3,900, 313, and 276. The transfers were incubated for 8, 16, 21, and 20 days, respectively. Calculated peptone concentrations were the same as in series VIII. The results (Table 1) showed growth in the first transfer, none in the second, growth in the third, and none in the fourth. Because of the low concentration of flagellates in the fourth transfer, the series was discontinued.

At this point a series of viability tests was carried out on old cultures at various transfers in media EA and EAB. At intervals tubes of peptone medium were inoculated in 1.0-cc. amounts from the cultures in inorganic medium and then incubated at room temperature. The results showed that *E. gracilis* was still viable in medium EA after 49 days in the first transfer, 36 days in the second, 19 days in the third, and 30 days in the fourth transfer. No attempt was made to determine the maximum in any case. The results for medium EAB showed survival for 30 days in the first transfer, 14 days in the second, and 26 days in the third. Obviously, neither of these media was lethal, since the flagellates survived at least in small numbers even in those transfers which showed no growth. These results led us to concentrate the flagellates before inoculating the third and fourth transfers in series X, in the hope of carrying over enough viable flagellates to maintain growth in the transfers.

Series X (*medium EAB*).—In different transfers the initial pH ranged from 6.7–7.0. Initial counts in the first twelve transfers were as follows: 2,307, 5,460, 700, 217, 1,573, 987, 360, 233, 900, 520, 553, and 813. Incubation periods were 14 days in the first, 20 in the second, and 21 days in subsequent transfers. The calculated peptone concentration was reduced from 10^{-4} in the first transfer to 3.5×10^{-19} in the twelfth transfer. Before inoculation of the third transfer, 1.0 cc. of concentrated material from one second-transfer tube was added to another, and the latter was used as stock for the third transfer. For the fourth transfer a similar procedure was followed. This modification of technique was introduced for the purpose of increasing the size of the initial count. The results (Fig. 1) showed good growth in the first transfer, poor growth in the second, none in the third, excellent growth in the fourth, and fairly good growth in all succeeding transfers. The series is now in the fifteenth transfer, with no marked change in growth-rate.

The first four transfers in this series proved to be interesting. In the second and third transfers it was noted that the majority of organisms were etiolated and otherwise abnormal in appearance, while a few were comparatively large, densely green, and quite active. In the fourth transfer the change was striking. Except for rare specimens, all of the flagellates were bright green and healthy in appearance. This type has been predominant in all the later transfers. The same phenomenon had been observed previously in series VI (Table 1), a series which was discontinued in the fourth transfer. These observations suggest a selective action of inorganic media in which many of the flagellates die in the first few transfers. The survivors, on the other hand, appear to be capable of continued growth in such media.

Series XI (*medium EF*).—The initial pH ranged from 6.6 to 7.5 in different transfers; and the initial counts in the first twelve transfers were: 2,147, 1,407, 147, 1,067, 567, 233, 540, 387, 513, 647, 340, and 320. The incubation period was 21 days in each case. Moderate growth was noted in the first transfer, very little in the second, excellent growth in the third, and moderate growth in subsequent transfers (Fig. 1). The calculated peptone concentration was reduced from 10^{-4} in the first transfer to 5×10^{-19} in the twelfth. Medium EF thus appears to be adequate for autotrophic nutrition of *E. gracilis*.

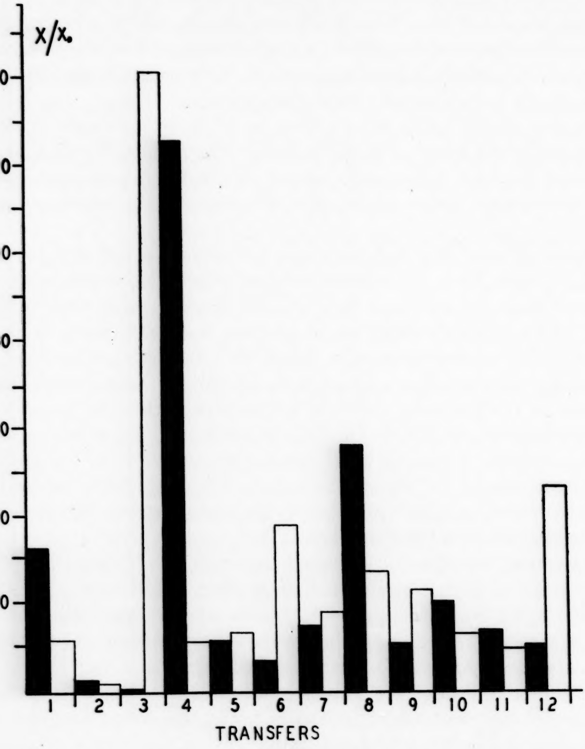

FIG. 1.—Series X and XI. Growth in successive transfers is expressed as x/x_0 (ratio of final to initial concentration of flagellates per cubic centimeter); series X in black, series XI in white blocks.

This series was similar to series X, except that the recovery in growth-rate occurred in the third transfer instead of the fourth. The same contrast in appearance of the two types of flagellates was noted in the second transfer of series XI, and practically all of the flagellates observed in the third transfer were normal in appearance. It was decided at this time to start several side series from series X. It seemed probable that, if a real selection of autotrophic forms had occurred, such flagellates would continue to grow moderately well in other inorganic media. Accordingly, series XII, XIII, and XIV were inoculated each from a fourth transfer tube of series X.

Series XII (*medium EC*).—The initial pH ranged from 6.9 to 7.2 in different transfers; and initial counts in successive transfers were: 1,427, 487, 427, 460, 400, 853, 347, and 240. In the eighth transfer the calculated peptone concentration had been reduced to 4.5×10^{-19} grams per cubic centimeter. Each transfer was incubated for 21 days.

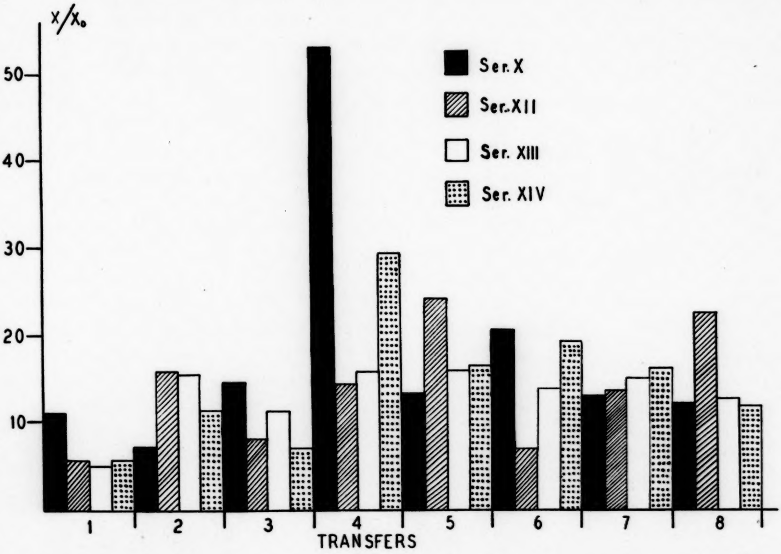

FIG. 2.—Series X (transfers 5–12, inclusive), and series XII–XIV (transfers 1–8, inclusive). Growth in successive transfers is expressed as x/x_0 (ratio of final to initial concentration of flagellates per cubic centimeter).

In the first transfer, run concurrently with the fifth transfer of series X, growth (Fig. 2) was about half as great as in the latter. In subsequent transfers, however, growth was sometimes greater and sometimes less than in the parent series. There was no indication of any selective effect.

Series XIII (*medium EA*).—This series was carried simultaneously with the fifth and later transfers of series X. Initial pH varied from 6.7 to 6.9; and successive initial counts were: 2,180, 987, 760, 613, 607, 880, 380, and 360. Calculated peptone concentrations were the same as in series XII, and the incubation period was also 21 days for each transfer. Growth in the first transfer (Fig. 2) was approximately the same as that in series XII, while growth in the succeeding transfers was usually comparable to that in the concurrent transfers of series X. In this series also there was no indication of any selection.

Series XIV (*medium EF*).—This series was started a week later than series XII and XIII. Initial pH ranged from 6.5 to 7.3; and initial counts were: 1,393, 960, 513, 307, 493, 647, 747, and 900. Each transfer was incubated for 21 days. Calculated peptone concentrations were approximately the same as in series XII. Growth in the first transfer (Fig. 2) was almost identical with that observed in series XII and XIII. Growth in subsequent transfers was fairly comparable to that in corresponding transfers of series X.

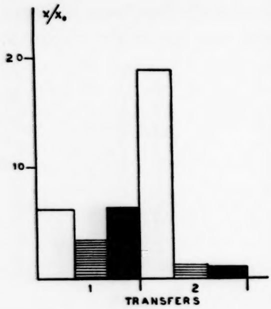

FIG. 3.—Growth of an autotrophic strain of *E. gracilis* for two transfers in medium EF (white blocks), Hutner's diluted medium (crosshatched blocks), and Hutner's concentrated medium (black blocks).

The results obtained in series XII–XIV are in accord with the assumption that a definite selection of flagellates capable of autotrophic nutrition occurred in the fourth transfer of series X.

Series XV.—This series was undertaken in an attempt to reconcile the findings of Hutner (1936) with those of Dusi (1933*a*) and the writers. Our medium EF, Hutner's (1936, p. 94) concentrated medium, and Hutner's diluted medium were compared in series inoculated from the same culture of one of our autotrophic strains in medium EF. All three sets of cultures were incubated under the same conditions at room temperature near a north window. In the first transfer the initial pH was 6.7 in each case; in the second transfer, 6.7 for medium EF and 6.8 for both of Hutner's media. Both transfers were incubated for 21 days. Growth (x/x_0) in the three types of media is described in Figure 3. It is obvious that our autotrophic strain of *E. gracilis* grew in both the first and second transfers in medium EF, while no growth occurred in the second transfer in either of Hutner's media and no normal green organisms were observed on microscopic examination of several samples. It thus appears that Hutner's media are much less satisfactory for growth of our autotrophic strains of *E. gracilis* than are the media used in series X–XIV.

Viability tests, as described above for media EA and EAB, were carried out on first and second transfer cultures in Hutner's media. In the case of Hutner's concentrated medium, growth was observed after 3 weeks in two out of three peptone cultures inoculated from first transfer tubes. However, no growth was obtained in peptone medium inoculated from second transfer tubes, and microscopic examination of centrifuged material showed no normal flagellates. Similar tests showed that *E. gracilis* remained viable for 7 weeks in first-transfer tubes of Hutner's dilute medium and for at least 3 weeks in the second-transfer tubes. Hutner's concentrated medium thus appears to be toxic to our autotrophic strains of *E. gracilis*, while his dilute medium still contains viable flagellates after several weeks, even though no population growth occurs.

DISCUSSION

The results described above are in agreement with the conclusions of Pringsheim, Mainx, and Dusi that *E. gracilis* is capable of carrying on photoautotrophic nutrition. Our failures in certain cases, and those of Hutner (1936), emphasize the difficulties of growing Euglenidae in inorganic media, in contrast to the comparative ease with which such a phytomonad as *Chlorogonium euchlorum* (Loefer, 1934; Hall and Schoenborn, 1938) may be established in such cultures.

After eight unsuccessful attempts the writers succeeded in establishing two autotrophic strains of *E. gracilis*. The conditions were somewhat interesting. Viability tests had shown that the flagellates remain alive for several weeks in certain media which apparently failed to support growth after the second or third transfer. In the hope of increasing the number of viable organisms in the inoculum, the flagellates were concentrated before inoculation of the third and fourth transfers in series X. Microscopic observations showed that in the second and third transfers a few of the organisms remained bright green and healthy, while the remainder were etiolated and otherwise abnormal. In the fourth and subsequent transfers the green type has been observed almost exclusively, and fair to excellent growth has occurred. In series XI, in which no concentration of flagellates was attempted, the same phenomenon was observed, with the exception that the green autotrophic population was established in the third transfer instead of the fourth.

These observations suggest the occurrence of a selective process in which those flagellates capable of autotrophic nutrition survived, whereas the rest died in the first few transfers. It may be assumed that our pure line of *E. gracilis*, which had been maintained in our own laboratory for more than 6 years in peptone media, contained a certain proportion of flagellates capable of carrying on photoautotrophic nutrition. Upon transfer to an inorganic medium these flagellates continued to multiply, whereas the remainder were unable to become adapted to the new conditions. Such an assumption may explain the rapid decrease in rate of population growth observed in the first few transfers of series X and XI.

If such a selective process had occurred, it might follow that the resulting autotrophic strain should be able to grow in other satisfactory inorganic media without any marked change in growth-rate. This hypothesis was tested by starting several series from the fourth transfer of series X in media EC, EA, and EF. These new series (XII–XIV) showed fair to moderate growth from the first transfer on, and there were no microscopic indications of selection. While the several series showed variations in growth in different transfers, such results were to be expected, since initial pH and initial counts varied and external conditions (light and temperature) were not constant throughout the period of observation. These preliminary observations thus suggest the occurrence, in certain inorganic media, of a definite selection which eliminated many of the flagellates in early transfers and permitted survival of a strain of *E. gracilis* capable of continued growth under conditions of photoautotrophic nutrition.

The occurrence of such a process may account for some of the contradictory results obtained in studies on autotrophic nutrition of Euglenidae. The combination of a process of selection and the progressive decrease in concentration of flagellates inherent in the technique of serial transfers would greatly increase the difficulties in such investigations. The writers were fortunate in obtaining positive results in two out of ten series started from stock cultures in peptone media. Hutner (1936) did not depend upon microscopic observations for measurement of growth, and thus may have overlooked significant changes such as those observed in the early transfers of our series X and XI. In addition, the results obtained in series XV indicate that Hutner's concentrated medium is toxic to one of our autotrophic strains of *E. gracilis*, and his dilute medium is not favorable to growth of the same strain.

The case of *Chilomonas paramecium* may possibly be analogous to that of *E. gracilis*. Mast and Pace (1933) have reported that *C. paramecium* is capable of heteroautotrophic nutrition, while Loefer (1934), Lwoff and Dusi (1934), Pringsheim (1935), and Hall and Loefer (1936) have been unable to duplicate these results. Conceivably, the autotrophic strains of Mast and Pace might have been established as a result of selective adaptation, whereas other workers have been less fortunate in their investigations.

SUMMARY

The establishment of two autotrophic strains of *E. gracilis* is described. Establishment of the autotrophic strains involves, in early transfers, the death of most of the population, while a small proportion of facultative autotrophs becomes adapted to the inorganic media and multiplies to produce healthy autotrophic strains. One strain is now in the fifteenth transfer, and the other is in the thirteenth. One of the strains has been transplanted successfully into three different media, indicating that, once an antotrophic strain is established, it is capable of growth in any one of several satisfactory inorganic media. Attempts to grow an autotrophic strain in Hutner's (1936) media were unsuccessful.

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RELATION BETWEEN THE RESPONSES OF AMOEBA PROTEUS TO ALTERNATING ELECTRIC CURRENT AND SUDDEN ILLUMINATION[1]

(One figure)

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MANY organisms react in a definite manner to sudden changes in intensity of a stimulating agent—plants usually by a change in rate of growth, animals by a motor response of some kind. Reactions of this sort are usually referred to as "shock reactions." Among animals, the amoeba is known to be especially favorable for a study of shock reactions, as it reacts to a number of stimulating agents with a characteristic response, which consists of a cessation of protoplasmic flow. Direct electric current, alternating current, light, mechanical shock—all bring about a definite reaction in this animal.

It has long been known that the amoeba orients in a direct current, moving toward the cathode. Hahnert (1932) found that, if an amoeba is moving toward the anode as the current is turned on, it finally reverses its direction of movement, first stopping for a time, and that the time required to bring about this stoppage depends on the strength of the current, being shorter in a strong current than in a weak one. Mast (1931*a*) reports that the amoeba usually stops when subjected to an alternating current; and Thomas (1935, 1937), who studied the reaction in some detail, found that, as in the case of a direct current, the reaction-time is longer in a weak current than in a strong one. He found that the reaction-time consists of a stimulation period, during which exposure to alternating current is necessary if an amoeba is to respond and which takes up the first part of the reaction-time, and a latent period, during which exposure is not necessary for a response.

Folger (1925) had already determined the same phases in the response to light by the amoeba that Thomas later found to exist with alternating current. In both cases the amoeba ceased moving upon being stimulated, and the reaction-time became shorter as the intensity of the stimulus increased. In both cases the stimulation period became shorter as the intensity of the stimulus increased; the latent period showed an increase in the length and then a decrease as the intensity of the stimulus increased; the period of quiescence, that is, the time during which the amoeba remain inactive, was found to vary directly as the intensity increased; each reaction is followed by a refractory period, during which there is a decrease in the sensitivity of the animal to stimulation.

Folger (1926) discovered that the responses to mechanical shock are also very much like those to light. Working with light and mechanical shocks, Folger (1927) found that a very close relationship exists between the two. This relationship is demonstrated in his significant finding that an amoeba which has just responded to sudden increase in luminous intensity is rendered less sensitive not only to illumination but also to mechanical shock. The reverse of this was also found to be true.

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[1] Contribution from the Biology Laboratory of Fisk University. The writer wishes to express his gratitude to Professor H. T. Folger, under whose direction the work was done, and to Professor Elmer S. Imes, of the physics department of Fisk University, for invaluable assistance with the electrical equipment.

From the foregoing statement it is to be seen that, when used singly, sudden illumination, alternating current, and mechanical shock produce similar effects on the amoeba. As pointed out, a close interrelationship exists between the response to light and the response to mechanical shock. This paper is concerned with experiments designed to find out if there is a similar relationship between the reactions to light and to alternating current.

MATERIALS AND METHODS

All the observations described in the following pages were made upon specimens of *Amoeba proteus* that were cultivated in glass bowls containing about 100 cc. of a culture solution made by adding raw hay and wheat to distilled water. The hay and wheat soon became covered with a mold, on and among the strands of which aggregated numerous specimens of bacteria and small ciliates, upon which the amoebae fed.

In preparing the amoebae for observation, from ten to twenty of the most active specimens were transferred, by means of a capillary pipette, from the culture solution to a finger bowl of distilled water. They were left in this bowl 15–30 minutes. By this time nearly all of them were radiate in form. The radiate ones were removed and transferred directly to the test solution, which was a modified Ringer's solution devised and described by Chalkley (1929). The experiments were carried on in a darkened room, where the temperature varied little during any experiment.

The electricity used was drawn from a 110-volt alternating-current lighting circuit wired in parallel with a rheostat (G) of 922 ohms resistance (see Fig. 1). A glass trough (H) was made by cementing thin strips of glass on the surface of a glass slide $2\frac{1}{2}$ inches long and $1\frac{1}{2}$ inches wide. This trough was placed in a wood frame (K) of similar inside dimensions on the stage of a compound microscope (N), at each end of which was a metal binding post (J), to which a platinum electrode (I), $1 \times \frac{1}{2}$ inch in size, was attached. These electrodes rested evenly on the bottom of the trough in which were the amoebae to be observed.

By connecting the slide contact (F) of the rheostat to one of the electrodes over a key (L) and by connecting one of the other two binding-posts of the rheostat directly to the second electrode, as potential divider arrangement was obtained whereby the voltage on the electrodes could be varied at will. The resistance of the test solution was measured by a Wheatstone-bridge method of measuring electrical resistance and was found to be 20,560 ohms. From the equation $I - (E/R)$, where I is the current, E the voltage, and R the resistance, it is seen that the current will vary directly as the voltage E if the resistance R is kept constant. In the setup for the experiments to be described in this paper the resistance was constant, so that the current did vary directly as the voltage. Owing to the type of setup, it was found that variations in the voltage could be measured more accurately than variations in the current.[2] By dividing the rheostat into twenty-two equal parts and setting the slide contact on any one of these marks, the voltage could be increased in steps of 5 volts.

The apparatus for the experiments involving light (see Fig. 1) was very similar to that used by Folger (1925). Light was obtained from a 1,000-watt 123-volt Mazda stereopticon lamp (*B*), mounted in an asbestos-lined box (*A*). A beam of light, admitted through a circular aperture (*D*), 50 mm. in diameter, was reflected to the stage of the microscope by means of the substage mirror set at an angle of 45°. The light rays were passed through distilled water (*C*) for absorption of heat. The lamp was always turned on only a few moments before the shutter (*E*) was taken from in front of the circular aperture. This gave the lamp filament sufficient time to reach its maximum brightness before it was flashed on the amoebae in the observation trough.

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[2] When the entire rheostat was included in the setup, the current was approximately 0.005 amperes; and when it was excluded, the current was 0.05 amperes.

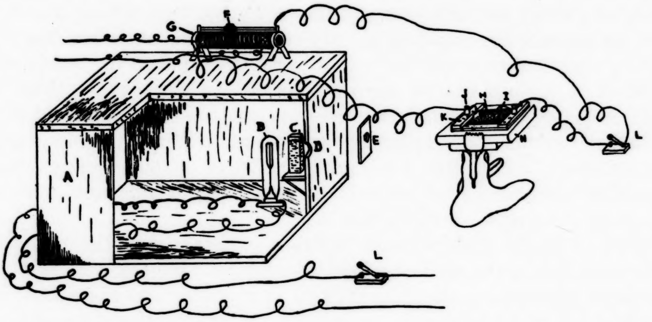

FIG. 1.—Diagram of apparatus used in experiments on amoeba. *A*, asbestos-lined box; *B*, stereopticon lamp; *C*, distilled water; *D*, circular aperture; *E*, shutter; *F*, slide contact of the rheostat; *G*, rheostat; *H*, glass trough; *I*, platinum electrode; *J*, metal binding-post; *K*, wood frame; *L*, switch key; *N*, stage of microscope.

Before an animal was experimented upon, either with light or electricity or the two combined, it was allowed to remain in the dark from ½ to 2 hours, so as to become adapted to the dark and acclimatized to the temperature of the room. A 7.5-watt red electric bulb in a lamp stand furnished sufficient light so that the amoebae might be examined at any time. This red light was equivalent to darkness, having no observable effect on the amoebae. The observations were made with various degrees of magnification, depending on the type of experiment being conducted. When the light was only 25 cm. from the trough, a piece of red glass was put over the ocular of the microscope to protect the eyes.

EXPERIMENTAL RESULTS

I. REFRACTORY PERIODS

a) *Variations in reaction-time after various refractory periods for light.*—Folger (1925) has shown that after an amoeba has been subjected to sudden increase in light intensity and has responded to this stimulus, a certain amount of time is required for recovery from the effects of the stimulus before the amoeba will again respond in the same manner to a second illumination. If the time for recovery is very short, there will be no response; if the time is somewhat longer, the amoeba may respond, but with an increased reaction-time. Ultimately, usually within 1½ minutes, the amoeba will have completely recovered and will again respond in the usual manner. The time during which the amoeba shows a decrease in sensitiveness to light stimulus is apparently analogous to the refractory period found in the response of nerve. During this time the animal is recovering from the effects of the stimulation. The general term "recovery period" has usually been used in this paper to indicate the time between stimulations. In the following preliminary experiment the work of Folger has been repeated and paralleled.

An actively moving amoeba, preferably monopodal, was brought to the center of the field of the microscope, which was so placed that the light was 24 cm. from the experimental trough. The animal was exposed to illumination; the reaction-time was obtained; and then, after a recovery period of 90 seconds, the amoeba was again stimulated and the reaction-time ascertained. Ninety seconds were found to be sufficient for complete recovery in every amoeba used, as shown by the fact that a recovery period of longer than 90 seconds produced no shorter reaction-time. After finding the reaction-time following complete recovery, one could begin the experiment proper, which was to show the effect of various shorter recovery periods on the reaction-time. A complete experiment consisted of (1) stimulation after 90 seconds for recovery, followed by (2) stimulation after various periods ranging from 60 seconds to 7.5 seconds and (3) ascertaining the reaction-time after each stimulation. Three or four readings were taken for the reaction-time after each recovery period and averaged. The results of three experiments of this type are presented in Table 1.

Examination of Table 1 shows that the results obtained in the various individual tests for the three specimens used were fairly consistent. In each case, decided increases in reaction-times were seen as the time allowed for recovery was shortened. Considering the results obtained with amoeba No. 2, one sees that its reaction-time after a 90-second recovery period was 1.86 seconds; after a 60-second recovery period, 3.7 seconds; after a 45-second recovery period, 4.33 seconds; and after a 15-second recovery period, 6.7 seconds. Further examination of the table shows that none of the three animals reacted after recovery periods shorter than 15 seconds. In the cases of a few amoebae experimented with, 60 and 45 seconds were found to be sufficient for complete recovery, as shown by the fact that the average reaction-time following 60 and 45 seconds was approximately the same as that following 90 seconds. The results for every amoeba used showed the same general result, though, as may be seen from the table, there was considerable variation in the average reaction-times of the several animals used.

From these results the following conclusion can clearly be drawn. A certain amount of time, specifically 90 seconds or so, is necessary for an amoeba to completely recover from the effect of stimulation by light. A shorter time for recovery results in an increase in reaction-time. If the time for recovery is made less than 15 seconds, the animal refuses to respond at all.

b) *Variations in reaction-time for alternating current after various refractory periods.*—In his work on the effect of alternating current on amoeba, Thomas (1935) did not ascertain in detail the effect of insufficient recovery on the reaction-time. As mentioned before, in various other respects, his results were similar to those obtained by Folger with light and corresponding types of experiments. One might expect, then, that the relation between reaction-time and recovery period would be similar for the two stimulating agents. Table 2 presents the results obtained from an experiment designed to ascertain whether or not this is true. The procedure for this experiment was identical with that for the one just described, except that stimulation by light was replaced with stimulation by alternating current. The voltage on the electrodes was kept at 75 volts, this voltage having been found to be the most favorable for the purpose of the experiment. A recovery period of 90 seconds was sufficient for all the amoebae to completely recover from previous stimulation by alternating current.

TABLE 1

Relation of Recovery Period and Reaction-Time for Light

Amoeba Number	Recovery Period Allowed (Seconds)	Average Reaction-Time (Seconds)
1	90	2.6
	60	3.3
	45	3.9
	30	4.57
	15	5.7
	7.5	No reaction
2	90	1.86
	60	3.7
	45	4.33
	30	4.83
	15	6.7
	7.5	No reaction
3	90	2.0
	60	3.6
	45	4.1
	30	4.2
	15	5.2
	7.5	No reaction

TABLE 2

Relation of Reaction-Time to Recovery Period for Alternating Current

Amoeba Number	Recovery Period Allowed (Seconds)	Average Reaction-time (Seconds)
1	90	1.1
	60	1.1
	45	1.2
	30	3.0
	15	11.0
	7.5	No reaction
2	90	1.15
	60	1.31
	45	1.8
	30	3.0
	15	8.35
	7.5	No reaction

From Table 2 it is seen that amoeba No. 2, for instance, gave a reaction-time of 1.15 seconds after 90 seconds for recovery. After 60 seconds for recovery, the reaction-time was 1.31 seconds; after 45 seconds, 1.8 seconds; after 30 seconds, 3.0 seconds; after 15 seconds, 8.35 seconds. There was no reaction after recovery periods shorter than 7.5 seconds.

The similarity between the results of this experiment and the preceding one, where light was used as the stimulating agent, is evident. In both cases an insufficient time for recovery brings about an increase in reaction-time.

II. THE RELATIONSHIP OF SUDDEN ILLUMINATION AND ALTERNATING CURRENT

a) Changes in reaction-time for stimulation by light after various refractory periods following stimulation by alternating current.—The two preceding experiments show most clearly that reaction-time and recovery period have the same relation for *A. proteus* whether the stimulating agent is sudden illumination or alternating current. As mentioned above, Folger (1927) found that a mechanical shock preceding sudden illumination influences the response to the latter. Insufficient recovery from mechanical shock, followed by sudden illumination, resulted in a longer reaction-time or no reaction at all. The reverse of this was also found to be true. Since responses in amoebae to sudden illumination and alternating current are so similar, two questions arise: Does sudden illumination affect the response to alternating current? If so, is the reverse true? The following two experiments are designed to answer these questions.

As may readily be seen from the description of the apparatus used for stimulation by light and alternating current, the two separate setups could easily be used together. In the first experment an amoeba was stimulated by alternating current over a voltage of 75 and was then allowed to recover from its effects for various lengths of time. At the end of these periods, which ranged from 90 to 7.5 seconds, it was exposed to light from the lamp, which was placed at a distance of 50 cm. from the experimental animal. Stimulation by alternating current was always preceded by a period of not less than 90 seconds, which, as we have already seen, permits complete recovery. Table 3 presents the results of such an experiment.

Examination of Table 3 shows that alternating current does have an effect upon the response in amoeba to sudden illumination but that this effect is diametrically opposed to that which mechanical shock has upon response to light, as maintained by Folger. From the results recorded for amoeba No. 1, one sees an average response to light of 1.83 seconds after 90 seconds for recovery. This reaction-time was one of the quickest noted in any amoeba for light after a complete recovery. Yet, as may be seen from Table 3, when this particular amoeba was stimulated by light immediately after a previous stimulation by alternating current, the reaction-time became even shorter. Stimulation by light following a recovery period of 60 seconds gave a reaction-time of 1.70 seconds; following a recovery period of 45 seconds, a reaction-time of 1.56 seconds; following a recovery period of 30 seconds, a reaction-time of 1.36 seconds; following a recovery period of 7.5 seconds, a reaction-time of 1.10 seconds. Further observation of the table reveals that amoebae Nos. 2 and 3 show exactly the same effect. The decrease in reaction-time with decrease in time allowed for recovery from the effect of alternating current is very marked in each experiment. Clearly, alternating current makes an amoeba more sensitive to light.

b) Changes in reaction-time for alternating current after various refractory periods from sudden illumination.—The following experiment was designed to ascertain what effect light has on the response to alternating current. The method of procedure was the reverse of that described for the preceding experiment. An amoeba was first exposed to light, placed at a distance of 25 cm., and then allowed to recover from its effects for various lengths of time, after which it was subjected to alternating current, the voltage on the electrodes being 60.

The results tabulated in Table 4 are typical of those obtained with numerous amoebae. The two amoebae considered were chosen because their reaction-times after complete recovery were about the same. As may be seen from Table 4, amoeba No. 1 gave a reaction-time of 2.5 seconds after 90 seconds for recovery. After 60 seconds for recovery, the reaction-time had decreased to 1.2 seconds; after 45 seconds for recovery, to 1.0 second; after 30 seconds for recovery, to 0.9 second; and after 15 seconds for recovery, to 0.7 second. Amoeba No. 2 showed the same sort of result.

TABLE 3

EFFECT OF ALTERNATING CURRENT UPON RESPONSE TO SUDDEN ILLUMINATION

Amoeba Number	Time after Stimulation by Alternating Current before Stimulation by Light (Seconds)	Average Reaction-Time for Light (Seconds)
1	90	1.83
	60	1.70
	45	1.56
	30	1.36
	15	1.25
	7.5	1.10
2	90	2.50
	60	2.26
	45	1.80
	30	1.50
	15	1.32
	7.5	1.03
3	90	2.76
	60	2.50
	45	2.20
	30	1.80
	15	1.50
	7.5	1.20

Thus, it is evident that, just as exposure to alternating current sensitizes the amoeba to light, so light sensitizes it to alternating current.

c) *Summation effects of stimuli below the threshold intensity.*—Folger (1927) found that, if an amoeba is stimulated by a light which of itself is insufficient to bring about a reaction, and then immediately after by a mechanical shock, also too slight to bring about a reaction alone, there may be a summation effect; the two subminimal stimuli, working together, bring about a reaction. This has been taken to mean that the effects of light and mechanical shock are the same. As demonstrated in the preceding pages, the relationship between the effects of light and alternating current are not the same as between light and mechanical shock. If an amoeba is subjected to mechanical shock, it must undergo a recovery before it will respond to illumination. On the other hand, if it is subjected to alternating current, not only does it not require a period of recovery before it will respond to light, but it responds even more readily than if it had not been stimulated by electric current. It should be of interest to note the effect of the two stimulating agents when used concurrently, each being made so small as to have no visible effect by itself.

TABLE 4

EFFECT OF SUDDEN ILLUMINATION UPON THE RESPONSE TO ALTERNATING CURRENT

Amoeba Number	Time between Stimulation by Light and Alternating Current	Average Reaction-Time for Alternating Current
1	90	2.5
	60	1.2
	45	1.0
	30	0.9
	15	0.7
2	90	2.4
	60	1.8
	45	1.4
	30	1.2
	15	1.0

TABLE 5

EFFECT OF ALTERNATING CURRENT UPON THE RESPONSE TO LIGHT BELOW THE INTENSITY THRESHOLD

Amoeba Number		Number of Trials	No Reactions
1	Sudden illumination alone	3	3
	Sudden illumination following immediately after shock by alternating current	8	3
2	Sudden illumination alone	6	6
	Sudden illumination following immediately after shock by alternating current	4	0
3	Sudden illumination alone	6	6
	Sudden illumination following immediately after shock by alternating current	6	1
Totals	Sudden illumination alone	15	15
	Sudden illumination following after shock by alternating current	20	4

Table 5 shows the results of such an experiment. In this experiment subminimal stimuli, both of light and alternating current, were employed, one being applied immediately after the other. As indicated, no reactions at all were obtained when either of the two types of stimuli were used alone. But when the two were used together, one immediately after the other, reactions usually occurred. When alternating current was used first, followed immediately by sudden illumination, sixteen reactions were obtained out of twenty trials. When sudden illumination was used first, followed immediately by alternating current, eleven reactions were obtained out of sixteen trials.

These results are not at all unexpected. However, their similarity with those of Folger, just referred to, is, perhaps, somewhat misleading. Apparently, light and mechanical shock have the same effect on amoebae. The evidence seems to show that, when an amoeba is subjected to a short period of illumination and then to a slight mechanical shock, its condition is just as it would have been if another short period of illumination had been substituted for the mechanical shock. From what has been given in the preceding experiments it is clear that light, followed by alternating current, does not have the same effect as a longer, single exposure to light. The amoeba is not affected by the alternating current in just the same way as by the light but, having been sensitized by the latter, responds to a current that is ordinarily below the threshold intensity.

DISCUSSION

Involving, as they do, a cessation of movement, any explanation of the reactions and relationships that have been under consideration must be concerned with the mechanics of amoeboid movement. According to Mast (1926), the essential structure of amoeba, so far as movement is concerned, consists of a liquid inner portion, designated the "plasmasol," which is surrounded by a more solid layer, the "plasmagel." A very thin layer, the "plasmalemma," surrounds the entire animal but, for our purposes, may be ignored. The plasmagel is elastic; and being subjected to a pressure, probably osmotic, by the plasmasol, is stretched. If it were of the same strength throughout, the amoeba would assume a spherical form. Ordinarily, of course, this is not true. Actually the plasmagel is usually unequal in strength at various points and is continuously changing in the living animal, resulting in the variety of shapes so characteristic of the amoeba. In a moving amoeba the plasmagel is very thin and weak at the anterior end, so that it is continuously bulging at this point.[3]

It is evident that a certain equilibrium must exist between internal pressure and the relative strength of the plasmagel at various points on the amoeba. Any changes, either in internal pressure or in the plasmagel, must alter this equilibrium. The effect of both light and alternating current (Mast, 1931*a* and 1931*b*), and presumably mechanical shock, is to coagulate the thin sheet of plasmagel at the end of the advancing pseudopodium and thus prevent it from being pushed forward. As a result, the animal ceases to move. But even after movement is resumed, the equilibrium which is at first established is different from that which existed before the animal was stimulated. Hence the refractory period. Gradually the old equilibrium is re-established; and when this occurs, the refractory period is at an end.

But what of the relationships between the various stimulating agents that have been noted? Mast gives evidence (1932, p. 5) for believing that light strengthens the plasmagel not only at the tip of the advancing pseudopodium but at all other points as well. Thus, the general effect of light is to increase the elastic strength of the plasmagel. Recovery from its effects, then, we may assume, includes a lowering in the strength of the plasmagel. It takes some time, even after the light has been turned off, for the plasmagel to return to its former condition. Until it does, the amoeba is less sensitive to stimulation by light. Assuming that mechanical shock has the same effect on the plasmagel that light has, it is evident that mechanical shock should make an amoeba less sensitive not only to another mechanical shock but to light as well. Likewise, light should make an amoeba less sensitive to mechanical shock. Electricity, however, has an effect in many respects different from that of light. If an amoeba is placed in a direct current but with its anterior end facing the anode, it reverses its direction of flow, the anterior end now facing the cathode. Thus, the current has caused coagulation of the plasmagel at the one end of the amoeba, but at the same time it has caused solation of the plasmagel at the other end. Mast (1931*a*) has noted a similar effect with alternating current. He says (1931*a*), p. 326):

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[3] Of course, other factors are concerned in amoeboid movement, but they need not concern us here. For further information the reader is referred to Mast's (1926) account.

> The evidence presented in reference to the effect on *Amoeba proteus* of both the direct and the alternating current indicates that the assertion . . . . that electricity gelates cytoplasm, is misleading, for it shows that if an electric current causes gelation in a cell it probably always causes simultaneous solation, each being confined to a portion of the cell.

Thus we may conclude that, while alternating current causes increase in strength of the plasmagel at some points on the amoeba, it causes a decrease at other points. The general effect is probably a lowering of the strength of the plasmagel. Recovery from the effects of alternating current, then, includes a general increase in the strength of the plasmagel. During the refractory period following alternating current the amoeba is less sensitive to alternating current; but, as the effect of the current is—in some respects, at least—opposed to that of light, it is more sensitive to light. Light, of course, should and does have the same effect on response to alternating current.

SUMMARY

1. Amoebae react both to light and to alternating current by a cessation of movement, which takes place after an appreciable reaction-time.

2. In both cases the reaction-time is dependent on the magnitude of the stimulus, becoming longer as the magnitude decreases.

3. In both cases the reaction is followed by a distinct refractory period, during which the amoeba is less sensitive to stimulation, as indicated by failure to respond a second time, or, if it does respond, by an increase in reaction-time. However, within a short time, usually within 90 seconds, the amoeba has completely recovered, and again responds as at first.

4. Folger has reported that, during the refractory period which follows response to light, the amoeba is less sensitive not only to light but also to mechanical shock. The reverse of this is also true. However, during the refractory period following response to light, an amoeba is not less sensitive, but more sensitive, to alternating current, and responds to the current with a reaction-time that is shorter than it would have been if the stimulation by electricity had not been preceded by exposure to light. Likewise, exposure to alternating current renders the amoeba more sensitive to light.

5. The effect that one of these stimulating agents has on the other is also shown by the use of subminimal stimuli. If the amoeba is subjected to one of these stimulating agents that is too slight in itself to bring about a reaction, it may respond if it has been sensitized by the other stimulating agent, even though that also is too slight to bring about a reaction.

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ERRATUM

In Volume XI, page 362, Table 1, last columns, change 10.50 ± 0.44 to 10.50 ± 0.27 and 3.98 to 2.42.

corrected
R.F.